

EU forum

The SAFE project: 'plant food allergies: field to table strategies for reducing their incidence in Europe' an EC-funded study

The true prevalence of food allergy as an IgE mediated reaction is still under discussion. Using apple as a model allergen source a multidisciplinary consortium worked together at developing various strategies for reducing the incidence of fruit allergies in an EC-funded project. Patient allergen profiles were established using in vitro and in vivo tests with respect to geographic area and mild or severe symptoms. Apple allergens (Mal d 1–Mal d 4) were characterised, variants identified, cloned and sequenced. These individual allergens were used to increase the sensitivity and specificity of diagnosis. Furthermore, they provided better prognosis of disease severity. RT-PCR and ELISA were developed for determining the allergen specific mRNA and expressed allergenic protein in a large number of apple cultivars. Similarly, changes in allergen characteristics from harvest through storage to processing and the impact of agronomic practices were investigated. Allergen genes were mapped on a molecular linkage map of apple. The biological function of Mal d 1 was studied using the RNA interference strategy. Finally, consumer attitudes in Northern, Central and Southern Europe were gauged on the acceptability of low allergen cultivars or a GMO and its impact on product quality.

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Allergy has been described as the epidemic of the 21st century, affecting up to 40% of the general population of the developed countries (1). There, the prevalence of allergic diseases like rhinitis and asthma has increased dramatically over the past decades. Although some studies have shown similar trends for food allergy (2, 3), this is certainly less well established. The limited availability of convincing proof is partly because food allergies were not yet studied longitudinally in as much detail as inhalant allergies. Maybe even more importantly, the poor quality of diagnostic procedures for food allergy has hampered determination of reliable prevalence data. In general, both sensitivity and specificity are low compared with diagnostics for respiratory allergies (4). A further complicating factor is that food allergy can have its origin in sensitization to inhalant allergens and in direct sensitization to food (5–8). Clinical presentation is usually quite different with the latter form of sensitization translating into a higher risk for severe symptoms (9, 10). Most current diagnostic tests do not distinguish between both forms of sensitization. For adequate treatment this distinction is of great importance. At present, the only treatment for food allergy is avoidance (11, 12). Avoidance can be problematic (hidden food allergens) and potentially lead to an unbalanced diet. Against the background of the increasing prevalence of allergies in Europe, the European Commission has realized that

improved diagnostic procedures and extension of therapeutic possibilities are urgently needed. Under the fourth and even more under the fifth Framework Program of the European Commission, several network activities (concerted actions) and research projects were funded to address the topic of food allergy (13, 14). One of these research projects was the SAFE project. This paper describes the background, aims and experimental design of SAFE (contract no.: QLK1-CT-2000-01394).

The full title of the project is 'Plant food allergies: field to table strategies for reducing their incidence in Europe'. For SAFE, apple was chosen as a model food although it does not rank among the most dangerous allergenic foods such as peanut, tree nuts, or shrimp. There were several reasons to choose apple for SAFE. First of all, fruit allergy in general has a high prevalence and apple is one of the most important allergenic fruits. This would allow inclusion of sufficient numbers of patients into the study. Fruits are important components of a healthy diet, and therefore avoidance can have a significant negative impact. Moreover, apple is an important crop for European agriculture. A broad variety of cultivars with a variable degree of allergenicity is grown in various European countries. Another important reason to choose apple was the observation that the clinical presentation of apple allergy appeared to be quite different across Europe, with mild symptoms in Central and Northern

Europe and severe symptoms in Southern Europe (7, 15). At the start of the project, the most important apple allergens had already been identified and methods for their production as purified natural and recombinant proteins were available. Taken together, apple appeared to be an ideal candidate to reach the aims of SAFE.

The consortium

The SAFE project was carried out by a multidisciplinary consortium from seven different European countries (Austria, Finland, Italy, the Netherlands, the United Kingdom, Spain and Switzerland). It included research laboratories with expertise in biomedical research, food chemistry, plant genetics and molecular and structural biology, as well as clinicians, fruit growers, social scientists, representatives from patients' organizations and a major fruit juice producer (see Table 1).

Apple allergens

It is well established that up to 70% of birch pollen allergic patients in Northern and Central Europe display predominantly mild allergic symptoms when eating plant foods such as apple, peach, tree nuts, celery or spices. Allergy to apple without sensitization to birch pollen is extremely rare in these areas. In contrast, in Southern Europe, where birch trees are virtually absent, allergies to apple and related *Rosaceae* fruits such as peach and plum are frequently severe and found in both pollen and nonpollen allergic patients (7). The difference in clinical

presentation is related to the molecular characteristics of the allergens recognized by IgE. Resistance to proteolysis in the gastro-intestinal tract is thought to be the most important parameter for an allergen's potential to induce severe symptoms (16, 17). Four apple allergens were studied in SAFE, two with established links to birch pollen sensitization (Mal d 1 and Mal d 4) (18, 19) and two without clear links to pollen sensitization (Mal d 2 and Mal d 3) (20–22).

The major apple allergen Mal d 1 is homologous to the major birch pollen allergen Bet v 1. Primary sensitization occurs via Bet v 1, resulting in cross-reactivity of IgE to Mal d 1. This allergen is exclusively linked to mild and local allergic symptoms together referred to as the oral allergy syndrome (23). Based on sequence similarity, this protein belongs to a family of pathogenesis-related proteins (PR 10) (24), that may function as a plant steroid carrier (25). A number of isoforms of Mal d 1 is present in the apple fruit (26–28). The diversity of this protein family is also reflected on the genomic level (29). Multiple gene loci were detected for Mal d 1 isoforms spread across the apple genome. Modelling of the structure of Mal d 1 on the basis of the solved structure of Bet v 1 revealed a very similar fold (Fig. 1) (25, 30). Surface-exposed amino acids involved in IgE-binding were identified for Bet v 1. This information was successfully used to design a hypo-allergenic mutant of Bet v 1.

The second apple allergen Mal d 2 is a thaumatin-like protein (TLP), representative of another family of pathogenesis-related proteins (PR5) (20, 22). A thaumatin-like protein has been identified as an allergen in a limited number of other foods like kiwi, cherry, grape and bell pepper (31–34). It also has been reported as an allergen in pollen of the Japanese cedar (35), but so far no cross-reactivity has been reported between TLP from fruits and from pollen. Mal d 2 and the other TLPs have 16 cysteine residues at conserved positions forming eight disulphide bridges. This results in a compact allergen that demonstrates extremely high resistance to proteolysis, making it a candidate allergen to be involved in the induction of severe food allergies.

The third allergen is Mal d 3 or apple nonspecific lipid transfer protein (LTP), a member of the PR-14 family of pathogenesis-related proteins (17, 36–40). As in case of Mal d 2, LTP has several conserved disulphide bridges ($n = 4$) making it a very stable allergen. Again, this structural characteristic has been proposed to give the allergen a high potential to induce severe symptoms. LTP has been shown to accumulate in the peel of the apple fruits (7, 41). Cross-reactive LTPs have been reported in many fruits, nuts, cereals and vegetables, but with variable degrees of clinical relevance (42–44). Some pollen has also been shown to contain allergenic LTPs. The major allergens of *Parietaria* pollen, Par j 1 and Par j 2, are LTPs but cross-reactivity to foods does not seem to play a role (44). A limited degree of cross-reactivity has been demonstrated between fruit and

Table 1. Partnership of SAFE

Partner	Members	Country
Universität Wien (co-ordinator)	K. Hoffmann-Sommergruber/ H. Breiteneder/Y. Ma/ B. Bohle/C. Hafner	AT
European Federation of Asthma and Allergy	E. Valovirta	FIN
Institute of Food Research	C. Mills/A. Sancho/S. Miles N. Rigby/J. Jenkins	UK
Sanquin Research	R. van Ree/L. Zuimeer/ W.A. van Leeuwen	NL
Ospedale Caduti Bollatesi	R. Asero	IT
Plant Research International	L. Gilissen/E. van de Weg/ G. Zhongshan	NL
Universität f. Bodenkultur	M. Laimer/G. Marzban/ H. Puehringer	AT
CIV Consorzio Italiano Vivaisti	A. Martinelli	IT
Fundación Hospital Alcorcón	M. Fernandez-Rivas/ E. Gonzalez-Mancebo	ES
Rauch Fruchtsäfte GmbH	W. Schwald	AT
University Medical Centre Utrecht	A. Knulst/S. Bolhaar	NL
Norfolk Fruit Growers Ltd	T. Browne	UK
Universitätszentrum Basel	Z. Housley	CH

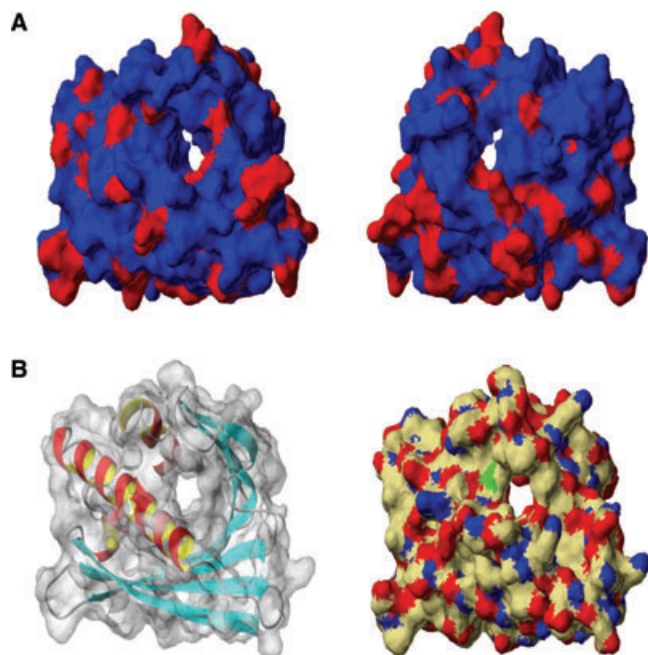


Figure 1. Molecular model of Mal d 1 and Bet v 1. (A) Front and back surfaces of the Mal d 1 model, coloured blue where the nearest atom to the surface is conserved and red where it varies within the Mal d 1 sequences. (B) Bet v 1 structure with helices and sheets shown with a transparent surface to the left and the surface coloured using the nearest atom on the right. Oxygens, red; nitrogens blue; carbons, khaki; sulphur, green to indicate hydrophobic or hydrophilic surfaces.

mugwort and plane pollen LTP (21, 45–48), but the consensus is that fruit LTP sensitization occurs by the oral route.

The last allergen studied in SAFE was Mal d 4 or profilin. It was first described as a minor allergen in birch pollen (Bet v 2). Sensitization to profilin is mediated by pollen (49, 50). There are no reports of IgE antibodies to profilins independent from pollen sensitization. The clinical relevance of profilin as a food allergen has been a matter of some debate. Again, the clinical presentation appears to be different depending on the geographical background of the patients (9, 51–54).

Food allergy diagnostics

Diagnostic tests for food allergy frequently have poor sensitivity and specificity (4) and therefore the double blind placebo controlled food challenge is generally regarded as the golden standard of the diagnosis of food allergy (55–57). This is in particular true for fruit allergy. Commercial skin test extracts used for skin prick test have poor sensitivity due to lability of major allergenic components resulting in false negative diagnosis (58, 59). The main reason is that major fruit allergens lose their

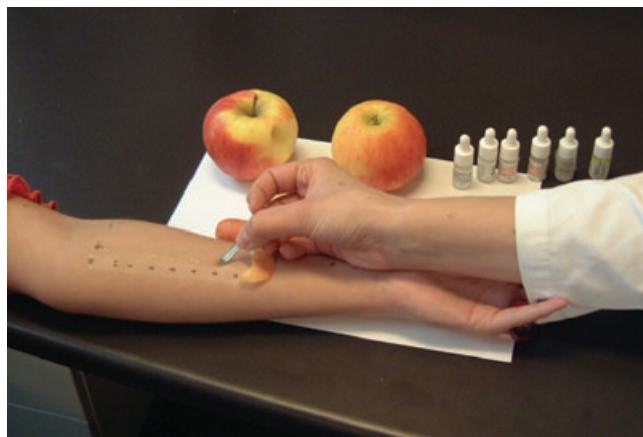


Figure 2. Skin prick test using the prick-to-prick method.

IgE-binding capacity upon disruption of the fruit tissue and subsequent extraction (60). Endogenous enzymatic activity is responsible for this decrease in quality. For this reason most clinicians use the prick-to-prick method with fresh fruits for skin testing (Fig. 2). Obviously, standardization of this procedure is impossible and availability of fresh fruits can be problematic. Extracts for *in vitro* tests can be protected from enzymatic attack by addition of inhibitors that are not compatible with *in vivo* use. Therefore, sensitivity of *in vitro* diagnostics is usually not a problem. Here specificity is the main issue. In particular IgE antibodies against cross-reactive structures like profilins or carbohydrate determinants can lack biological activity resulting in false-positive diagnosis (54, 61, 62). Purified major (and minor) allergens are protected against enzymatic attack and potentially facilitate distinction between clinically relevant and irrelevant IgE responses. Their use in the diagnosis of food allergy can also help elucidate the origins of sensitization.

Therapeutic options

At present the only therapy for food allergy is avoidance. Complete avoidance of apple and related fruits and nuts like pear, peach, cherry and hazelnut deprives the patient's diet from important sources of vitamins, minerals and fibres. Some patients claim that they only experience adverse reactions if they eat the fruit with the peel. Knowing the distribution of individual allergens over apple tissues can provide a scientific explanation for these observations. From growth and harvest to consumption apples are (possibly) exposed to pesticides, picked, transported, stored and sometimes processed. All these external influences can have impact on the allergenicity. Characterization of these effects is essential for designing strategies to reduce allergenicity. Largely anecdotal data further suggest that there are significant differences in allergenicity between apple cultivars. Some



Figure 3. Transgenic apple shoots low in Mal d 1.

patients claim to tolerate certain cultivars whereas other apples give clear symptoms. The identification and characterization of the main apple allergens offer the possibility to elucidate the basis of differences in allergenicity among cultivars. In addition, this know-how might be used by apple growers to develop novel cultivars with decreased allergenicity. Alternatively, molecular biological techniques like RNA-interference can be used to knock out major allergens like Mal d 1 (Fig. 3).

Allergen specific immunotherapy is successfully used for the treatment of respiratory allergies including birch pollen allergy (63). It is a longstanding debate whether birch pollen immunotherapy is beneficial for the cross-reactive fruit and nut allergies. The availability of purified major allergens from birch pollen and apple offers the possibility to study the role of cross-reactivity in immunotherapy at the molecular level. One of the problems of allergen-specific immunotherapy is the risk of anaphylactic side-effects (64). The use of site-directed mutagenesis to produce safer hypo-allergenic mutants of major food allergens can be tested using the structural knowledge available for Bet v 1 and Mal d 1.

The aims and experimental design of SAFE

The full title of the SAFE project 'Plant food allergies: field to table strategies for reducing their incidence in Europe' indicates that the project's major aim was to design strategies to reduce the incidence of food allergy, using apple allergy as a model. Apple allergic patients are not a homogeneous group: pollen-related *vs* isolated food allergy, mild *vs* potentially life-threatening severe symptoms, allergic to fresh and processed or to fresh apple only, from Northern and Central or from Southern Europe, etc. Strategies to reduce apple allergy will have to take such differences into account. It was therefore pivotal to the project's success to include a large panel of apple allergic patients with differing clinical and

geographical backgrounds. To this end ~100 patients were included at four different locations: Madrid (Spain), Milan (Italy), Vienna (Austria) and Utrecht (The Netherlands). The aim was to fully characterize the specific IgE spectrum against Mal d 1, 2, 3 and 4. These allergens had to be produced as purified natural and/or recombinant allergens by the collaborating research laboratories. To be able to characterize the allergenicity of different apple cultivars and the influence thereon of growth conditions, harvest and postharvest handling, fruit growers from the Norfolk (UK), Wageningen (The Netherlands) and Ferrara (Italy) were involved in the project. The allergenic make-up of different apple cultivars was to be evaluated both at genetic and protein level by research laboratories with expertise in plant genetics and protein chemistry, respectively. The latter groups together with a major fruit juice producer also set out to study the influence of different processing steps on the structure and immune-reactivity of the major apple allergens. Finally, SAFE also aimed at investigating the potential of RNA-interference to produce a hypo-allergenic apple (Fig. 3). In parallel, the degree of acceptance of such a biotech apple as well as the allergen labelling aspects of the general public and allergic patients were studied by demographic methods (65, 66). Summarizing, the aims of SAFE were the following:

1. Characterize differences across Europe in sensitization to apple allergens and the resulting clinical expression.
2. Characterize the spectrum of IgE antibodies against plant foods and pollen to identify the most likely route of sensitization that gave rise to apple allergy.
3. Develop novel diagnostics using purified apple allergens that will increase sensitivity and specificity as well as provide better prognosis for disease severity.
4. Develop methods to characterize the genetic and allergenic make-up of apples.
5. Evaluate differences in allergenicity of different apple cultivars to provide breeders and consumers with relevant information.
6. Analyse the influence of growth conditions, harvest and postharvest handling as well as processing steps on allergenicity.
7. Evaluate the potential of RNA-interference for the development of a hypo-allergenic apple.
8. Investigate the degree of acceptance by general public and patients of a GMO with a consumer benefit (hypo-allergenicity).
9. Investigate the effect of birch pollen immunotherapy on cross-reactive apple allergy.

The outcome

The SAFE project was performed between 1 January 2001 and 31 December 2003. Most of the aims were

reached. A group of 396 apple allergic patients from Spain, Italy, Austria and The Netherlands was studied in detail both clinically and serologically. Clear geographical differences in allergen recognition were observed, with Mal d 1 domination in Northern and Central Europe and Mal d 3 in Spain and to a lesser extent in Italy. Recognition of Mal d 1 was exclusively accompanied by mild symptoms whereas Mal d 3 was identified as a risk factor for more severe systemic reactions. These observations will form the basis for the development of improved diagnostics based on purified major allergens. RT-PCR and allergen ELISA were developed for quantifying allergen-specific mRNA and expressed allergenic protein. These were used to screen >80 cultivars for allergenicity. Clear differences in allergenicity were observed both for Mal d 1 and Mal d 3. The proof of principle to develop a hypo-allergenic apple by RNA-

interference (67) was delivered (68). By interview perception of such a biotech apple was collected among allergic patients. Hypo-allergenicity of a mutant of Mal d 1 was confirmed by oral provocation in apple allergic patients. This offers perspective of safe immunotherapy for food allergy. Last but not least birch pollen immunotherapy was shown to have a beneficial effect for apple allergy as well (69). At present several manuscripts have been published or accepted for publication, and many others are still to come. The SAFE project has successfully generated new data that can help to reduce the burden of food allergy. The multidisciplinary network of SAFE, together with other successful networks funded under the fourth and fifth Framework Program, now form the basis for a successful application for an Integrated Project (EuroPrevall) in the field of food allergy under the sixth Framework program.

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