Invited Review

Awareness and knowledge of allergens: A need and a challenge to assure a safe and healthy consumption of small fruits

Margit Laimer* and Fatemeh Maghuly

Plant Biotechnology Unit, IAM, Department of Biotechnology, VIBT BOKU, Vienna, Austria

Abstract. Food allergy is a widespread phenomenon: An estimated 1–2% of the population suffers from some type of food allergy. Fruits and vegetables are considered among the most important elicitors of food allergy. Berry fruits rich in phenolic compounds including flavonols, flavones and anthocyanins have recently gained increasing interest due to their possible beneficial effects on human health. However, they may harbour a series of allergenic proteins that cause discomfort or even represent serious threats to certain individuals. The identification and characterization of allergens in fruits like berries from distant taxa, requires novel approaches involving genomic and proteomic tools. The allergen content of blueberries (*Vaccinium myrtillus*), strawberries (*Fragaria ananassa*), raspberries (*Rubus idaeus*), and blackberries (*Rubus fruticosus*) was determined. Different extraction procedures are required to improve protein detection with polyclonal and monoclonal antisera raised against different allergens from *Malus domestica* in Western blotting. Due to the cross-reactivity they are able to recognize similar epitopes in highly conserved protein families of other plant species. Fruit extracts were analysed by two dimensional electrophoresis and mass spectrometry. Using clinical screening tools from a high number of patients and patient sera delivered conclusively results on allergenic proteins present in the small fruits.

Keywords: Small fruit allergens, non-specific lipid transfer proteins (nsLTPs), pathogenesis-related proteins, thaumatin like proteins (TPLs), strawberry, raspberry, blackberry, blueberry, IgE-reactivity, Fra a 1, Fra a 3, Rub i 1, Rub i 3, class III acidic chitinase, cyclophilin, profilin

1. Fruit allergy

Fruits and vegetables are considered among the most important elicitors of food allergy in humans. Food allergy is a widespread phenomenon: An estimated 1–2% of the population (up to 8% in children) suffers from some type of food allergy [20]. Food allergy is generally an IgE-mediated reaction, and is the result of a sensitization process generally occurring in the gastrointestinal (class 1 allergy) and/or respiratory (class 2 allergy) tracts [31]. Allergic patients produce specific IgE antibodies after frequent exposure to plant food allergens, accompanied by clinical symptoms ranging from pruritus, swelling of the lips, tongue and oral mucosa (oral allergy syndrome OAS), itching, cough and pruritus of the ear canals to gastro-intestinal symptoms, rhinitis, asthma, skin reactions, and the most severe reaction, systemic anaphylaxis. Exposure to inhaled plant allergens occurs mainly through pollen, spores, and

*Corresponding author: Margit Laimer, Plant Biotechnology Unit, IAM, Department of Biotechnology, VIBT BOKU, 1190 Vienna, Austria. Tel.: +43 136006 6560; Fax: +43 13697615; E-mail: margit.laimer@boku.ac.at.

plant-derived products such as cosmetics and rubber articles, that may cause rhinoconjunctivitis, asthma, oedema, urticaria and anaphylaxis [25].

2. Which are the known plant food allergens?

A protein can be described as a new allergen, if a minimum of 6 case reports have been documented. Allergens are designated according to the accepted taxonomic name of their source as follows: The first three letters of the genus, space, the first letter of the species, space, and an Arabic number [29]. Allergenic epitopes are divided in two main classes: (i) Conformational epitopes are expected to be more susceptible to processing-induced changes and (ii) linear epitopes, likely to be more resistant to physical treatments and to be deactivated only by hydrolysis [1].

The International Union of Immunological Societies Allergen Nomenclature Subcommittee (http://www.allergen.org) proposed to classify plant food allergens into families and superfamilies on the basis of their structural and functional properties. In fact, most plant food allergens belong to a few protein families and superfamilies. The most widespread groups of plant proteins that contain allergens are the cupin superfamily (7S and 11S seed storage proteins) or the prolamin superfamily (2S albumins, nonspecific lipid transfer proteins [nsLTPs], α-amylase/trypsin inhibitors, and prolamin storage proteins of cereals) and the protein families of the plant defence system [9, 10].

Of the plant allergens listed in the Official Allergen Database of the International Union of Immunological Societies, a considerable number belong to the group of pathogenesis-related proteins (PR-proteins). PR-proteins are defined as proteins that are induced upon stress, pathogen attack and abiotic stimuli. This inhomogeneous group of proteins has been classified into 17 PR-protein families (Table 1). So far, plant-derived allergens have been identified with sequence similarities to PR-protein families 2, 3, 4, 5, 8, 10 and 14. In general, they represent rather small proteins, which are stable at low pH and resistant to proteolysis. These features, and their level of expression, make PR-proteins good candidates for evoking an immune response in predisposed humans, when coming into contact with mucosal surfaces [23]. In addition, there are some unrelated families of structural and metabolic plant proteins that harbor allergenic proteins, such as the profilins [54].

Table 1

Pathogenesis related proteins found in plants [33]. Members of the PR-3, PR-5, PR-8, PR-10 and PR-14 families, also known to be allergens are highlighted

Family	Type member	Properties	Gene symbol
PR-1	Tobacco PR-1a	Antifungal	*Ypr1*
PR-2	Tobacco PR-2	b-1,3-glucanase	*Ypr2*, [*Gns2* ('*Glb*')]
PR-3	Tobacco P, Q	Chitinase type I, II, IV, V, VI, VII	*Ypr3, Chia*
PR-4	Tobacco 'R'	Chitinase type I, II	*Ypr4, Chid*
PR-5	Tobacco S	Thaumatin-like	*Ypr5*
PR-6	Tomato Inhibitor I	Proteinase-inhibitor	*Ypr6, Pis* ('*Pin*')
PR-7	Tomato P69	Endoproteinase	*Ypr7*
PR-8	Cucumber chitinase	Chitinase type III	*Ypr8, Chib*
PR-9	Tobacco 'lignin-forming peroxidase'	Peroxidase	*Ypr9, Prx*
PR-10	Parsley 'PR1'	'Ribonuclease-like'	*Ypr10*
PR-11	Tobacco 'class V' chitinase	Chitinase, type I	*Ypr11, Chic*
PR-12	Radish Rs-AFP3	Defensin	*Ypr12*
PR-13	Arabidopsis THI2.1	Thionin	*Ypr13, Thi*
PR-14	Barley LTP4	Lipid-transfer protein	*Ypr14, Ltp*
PR-15	Barley OxOa (germin)	Oxalate oxidase	*Ypr15*
PR-16	Barley OxOLP	'Oxalate oxidase-like'	*Yrp16*
PR-17	Tobacco PRp27	Unknown	*Yrp17*

2.1. PR-5 family: Thaumatin-like proteins (TLPs)

Thaumatin is an intensely sweet-tasting protein from the African shrub *Thaumatococcus daniellii* [23]. Based on sequence similarity, all PR-5 proteins are designated TLPs, although none has a sweet taste. TLPs contain 16 cysteine residues forming eight disulphide bridges. TLPs have been detected in the leaves of young plants, but they accumulate rapidly to high levels upon biotic or abiotic stress, where they might be involved in causing osmotic rupture of the pathogen [57].

Members of the PR-5 family represent food allergens that are present in various plant-derived foods (Table 2). An apparent 31 kDa major apple allergen whose N-terminal sequence shares 46% identity with PR-5 proteins was the first TLP to be described as an allergen [24]. Mal d 2 was identified and characterised as a 23 kDa protein, although in most studies Mal d 2 migrated as a 31 kDa protein in SDS-PAGE [24]. The full cDNA sequence has been obtained and the allergen has received the designation Mal d 2 (http://www.allergen.org). Immuno tissue printing of apple fruit has shown the equal distribution of Mal d 2 in peel and pulp [39]. Mal d 2 has two interesting characteristics: (1) altered electrophoretic mobility in the gel, which was attributed to the presence of reducing agents [21] and (2) higher IgE-reactivity after reduction.

Due to their compact structure, Mal d 2 molecules were predicted to be extremely stable and therefore involved in the induction of severe food allergies [10], but contrary to previous assumptions, the current findings suggest that the allergenic epitopes of Mal d 2 are hidden inside the protein structure and none of the rigorous conditions applied in industrial juice processing or digestive proteolysis enhance or reduce the binding to IgE molecules [40].

2.2. PR-8 family: Class III chitinases

In animals and plants, chitinases are mainly involved in the defence against pathogen attack, however, a role in growth and developmental processes has also been suggested [28]. Plant chitinases can be divided into seven classes, with class III chitinase, showing no hevein domain [23, 32]. Class III chitinases are classified as PR-8 proteins and were originally described as lysozymes, as they have an additional lysozyme-like activity [23].

Table 2

Plant allergens show conserved structures in distantly related taxa. Allergens found in fruits and vegetables of different families display high sequence and conformational homology

Allergen families	Apple allergen	Other fruit, vegetable and important pollen allergens
PR-5 (TLP)	Mal d 1	Alder (Aln g 1), apricot (Pru ar 1), birch (Bet v 1), blueberry (Vac c 1), cherry (Pru av 1), chestnut (Cas s 1), hazel (Cor a 1), hornbeam (Car b 1), celery (Api g 1), kiwi (Act c 8), parsley (Pet c 1), peach (Pru p 1), pear (Pyr c 1), raspberry (Rub i 1), St. John's wort (Hyp p 1), strawberry (Fra a 1)
PR-10	Mal d 2	Bell pepper (Cap a 1), cherry (Pru av 2), grape (Vit v TLP), kiwi (Act c 2), mountain cedar (Cup a 3, Jun a 3), peach (Pru p 2)
PR-14 (LTP)	Mal d 3	Almond (Pru du 1), ambrosia (Amb a 6), apricot (Pru ar 3), asparagus (Aspa o 1), barley (Hor v 14), blueberry (Vac m 3), celery (Api g 2), cherry (Pru av 3), chestnut (Cas s 8), grape (Vit v 1), hazelnut (Cor a 8), kiwi (Act d 10), latex tree (Hev b 12), lemon (Cit l 3), maize (Zea m 14), olive (Ole e 7), orange (Cit s 3), peach (Pru p 3), peanut (Ara h 9), plum (Pru d 3), raspberry (Rub i 3), ragweed (Art v 3), soybean (Gly m1), strawberry (Fra a 3), sunflower (Hel a 3), tomato (Lyc e 3), walnut (Jug r 3), wheat (Tri a 14)
Profilin	Mal d 4	Alder (Aln g 2), avocado (Pers a 4), banana (Mus xp 1), bell pepper (Cap a 2), birch (Bet v 2), carrot (Dau c 4), cherry (Pru av 4), celery (Api g 4), goosefoot (Che a 2), grape (Vit v 4), hazelnut (Cor a 2), hornbeam (Car b 2), kiwi (Act d 9), latex (Fic c 4), lychee (Lit c 1), melon (Cuc m 2), mugwort (Art v 4), peach (Pru p 4), peanut (Ara h 5), pear (Pyr c 4), apple (Mal d 4), peanut (Ara h 5), persimmon (Dio k 4), pineapple (Ana c 1), soybean (Gly m3), strawberry (Fra a 4), tomato (Lyc e 1)

One of the major latex proteins from *H. brasiliensis*, hevamine, displays lysozyme and chitinase activity. Hevamine, a 30 kDa basic protein from the lutoids of *Hevea* latex, is involved in plugging the latex vessels and stopping latex flow. It has been identified as an allergen present in latex products, but it is regarded as a minor allergen from *H. brasiliensis*, and does not seem to play a role in the latex-fruit syndrome [23]. Recently a chitinase III has been reported as an allergen from Indian jujube, showing sequence homology with hevamine [32] and from raspberry [38].

2.3. PR-10 family: Intracellular proteins with unknown enzymatic function

Individuals with pollen allergy frequently have allergic symptoms after eating certain plant foods. The majority of these reactions are caused by allergens of *Rosaceae* fruits (e.g. apple, apricot, and pear) and *Apiaceae* vegetables (e.g. celery and carrot) that cross-react with allergens that are present in birch pollen, particularly the major birch pollen allergen Bet v 1, and other tree pollen (Table 2). Bet v 1 was the first allergen sequence published that showed homology to PR-10 family members [8]. It is noteworthy that all of these proteins are encoded by multiple genes (designated Ypr10) by van Loon and van Strien [63]. Most of the genes have been shown to be induced upon microbial attack [59], and by fungal elicitors [60, 68], wounding and stress stimuli [69]. As is the case with most of the other PR-protein families, PR-10-type proteins are also expressed in a tissue-specific manner during development. The presence of allergens in *Rosaceae* pollen was originally reported for a Bet v 1 homologous protein in apple tree pollen [6] and, the expression of Mal d 1, Mal d 3 and their homologues was confirmed by three independent methods ELISA, Western blot and RT-PCR [37].

Induction of Mal d 1 by pathogen and abiotic factors has been shown and a role in intracellular signalling has been suggested because of its ability to bind a novel apple protein, MdAP [52, 53]. Recently, intriguing data about the biologic activity of Bet v 1 and PR-10 proteins became available. Experimental evidence that Pru av 1 interacts with phytosteroids, and molecular modeling showed that the hydrophobic cavity of the protein is large enough to accommodate 2 such molecules [45].

Mogensen et al. [41] showed that Bet v 1 had an affinity for a number of ligands, including the plant pigments flavone and naringinin. Markovic-Housley et al. [34] provided evidence that suggested a plant steroid carrier function for Bet v 1 and other PR-10 proteins by showing the interaction of that Bet v 1 isoform with 2 brassinolide molecules.

2.4. PR-14 family: nsLTPs

As members of the prolamin superfamily [10], nsLTPs contain eight conserved cysteine residues forming four disulphide bridges, which makes them highly resistant to harsh temperature and pH changes [26]. nsLTPs are the most important allergens of the Prunoideae (e.g. peach, apricot, plum and cherry) when no pollinosis is involved (Table 2) [14, 15, 64]. nsLTPs can take part in plant defence, as some nsLTPs have potent antifungal and antibacterial activities [42]. LTPs may play a major role in membrane biogenesis by conveying phospholipids such as waxes and cutin from their place of synthesis to membranes that are unable to form these lipids. They usually accumulate in the outer epidermal layers of plant organs, thus explaining the stronger allergenicity of peels compared with pulps of *Rosaceae* fruits [10].

2.5. Profilins

Profilins are 12- to 15-kd cytosolic proteins that are found in all eukaryotic cells. They are present in almost all eukaryotic cells and are involved in the actin polymerization and the signal transduction of phosphatidylinositol pathway [55, 65]. Profilins are quite sensitive to heat denaturation and gastric digestion, and thus food allergy caused by profilin is usually confined to the oral allergy syndrome elicited by raw foodstuffs. The broad range of *in vitro* cross-reactivity, however, can not always be translated into clinical allergy and thus the clinical relevance of a profilin sensitization is still a matter of debate [72]. During recent years, several allergenic profilins from plant food sources were characterized, and their cDNAs were cloned (Table 2).

2.6. Newly identified classes of allergens

The number of allergens with known sequences continuously increases. New families of storage and structural proteins and metabolic enzymes are added to the already firmly established protein families that contain allergens.

Cyclophilins have been described as phylogenetically highly conserved proteins of approximately 18–20 kDa, present in mammalians, plants, insects, fungi and bacteria. All cyclophilins were found to have petidyl-prolyl isomerase activity [70]. This class of proteins has been attributed with a number of intracellular functions as chaperon-like and nuclease activity, involved in mRNA splicing [12]. Allergenic cyclophilins have been described in moulds, e.g. *Psilocybe cubensis, Aspergillus fumigatus, Malassezia furfur* and have been described as cross-reactive. The first allergenic cyclophilin from pollen was identified in birch, namely Bet v 7, which is recognized by about 20% of birch pollen allergic patients [11]. An allergenic cyclophilin was also reported in carrot and raspberry [16, 38]. Within the plant cyclophilins, cross-reactivity seems to be likely, leading to multi-sensitization patterns and probably pollen-food allergies [12].

3. Detection of allergens in fruits

Currently, ELISA is the most commonly used method for the detection and quantification of hidden allergens [50]. Specific monoclonal antibodies have already been produced for the detection of the major apple allergens Mal d 1 and Mal d 3 [35, 74]. Polyclonal antibodies are developed for the detection of Mal d 1, Mal d 3, Mal d 4 from apple, Pru p 3 from peach, Pru av 1 from cherry, Pru av 2 from cherry and allergens from golden and green kiwi, but there are still no antibodies or assays commercially available for the routine detection of fruit allergens in fresh and processed food [35, 58, 74]. ELISA methods for several fruit allergens have been developed and applied for the determination of cultivar dependent differences [35], an evaluation of the influence of ripening and storage on the allergen content in fruits [58], the detection of allergens in fruit products [18] and the assessment of allergen stability [59]. A series of serological *in vitro* assays exist which rely on patient sera, like radio-allergo-sorbent test (RAST), enzyme allergo-sorbent test (EAST), rocket immuno-electrophoresis (RIE), established for routine detection in clinical practice [50, 51].

Originally developed for the detection of plant viruses [30], immunoblotting is frequently used with the purpose to identify and characterize new IgE reactive molecules [27, 46–48]. IgE antibodies of sensitised patients are a valuable tool for the diagnosis and detection of allergens.

Proteomic studies of plant allergens and especially fruit allergens are still very rare, due to inherent obstacles of the fruit tissue for quality protein extraction [7] and sample preparation as crucial points for a successful two dimensional electrophoresis (2-DE) procedure [21, 56]. The preparation of protein samples from plant tissues is generally hampered by the low protein content, the high variability of the source material and the presence of high amounts of compounds, interfering directly with the electrophoretical process resulting in irreproducible results [2, 35, 38, 66].

Proteins in complex fruit extracts are separated by SDS-PAGE and subjected to immunoblotting using IgE antibodies [48]. The highest resolution can be achieved by 2-DE, which combines IEF and SDS-PAGE, allowing a separation according to the isoelectric point and the molecular weight [71]. The reactive protein spots can be identified by mass spectrometry (MS) and *de novo* peptide sequencing. Antibodies and purified proteins are applied in serological assays for routine analysis e.g. immunoblotting, enzyme-linked immunosorbent assay (ELISA) [5, 50].

4. Which allergens are known in small fruits?

Fruits like blueberry, raspberry and strawberry may be nutritionally underestimated, but scientific research shows that they may have huge impact on human health due to their high content of antioxidants and unknown biofactors, which play a role in ageing, cancer and infection prevention [4, 61]. Reports on allergenicity of small fruits such as strawberry, raspberry, blackberry and blueberry and elderberry are still rare. Whether this is related to a general low allergenicity, the small amounts consumed or the restricted time frame of consumption still remains to be answered.

As a matter of fact, low exposure to certain allergens might be the reason for the limited complaints recorded so far. However, with the ongoing encouragement for the consumption of small fruits, this situation might change. About 30% of patients reported hypersensitivity or adverse effects after consumption of strawberries, although many of these reactions might be related to food intolerance rather than food allergy [37]. Small fruits are not only consumed fresh but are also eaten as common ingredients in different food products as main or additional component e.g. jam, ice cream, cornflakes.

Already in 2005 attention was drawn to the fact, that fruit proteins with high primary sequence similarity display also homologous tertiary structures, resulting in similar epitopes to IgEs and consequently in cross-reactivity and therefore might pose an allergenic potential for pre-sensitised individuals also in strawberry, raspberry and blueberry, otherwise rich in beneficial biofactors [36]. Discussion about the nutritional and health related advantages of berries should at least mention the potential presence of risks factors for part of the consumer population, like allergens, as presented by Carbone et al. [13] in their paper dealing with molecular research in berry crops, focusing on antioxidant- and flavor-related compounds.

People vary in their reactivity to food and show a different pattern of reactivity depending on their individual characteristics. Individuals following specific diets tend to show a different pattern of allergic response. Clinical reactivity depends on a variety of factors including frequency of exposure to foodstuffs [60]. The interpretation of the phenomena depends strongly on the availability of patient independent detection tools and the sensitivity of the method. In many cases it is still unclear how results between *in vitro* assays, skin prick tests and oral challenge are correlated and what is the clinical importance of *in vitro* results. Will the patient, whose serum recognises a certain protein epitope develop an allergy in the near future? We actually do not know it, but we are developing tests to answer this in the near future.

From apple data we know that we have a high variability in Mal d 1-content among different cultivar, years and production systems. Analogous studies on small fruit allergens content are still under study. In Southern blot analyses DNA fragments homologues to Mal d 1 and Mal d 3 could be detected in genomic DNA from apple [53], strawberry, raspberry, blueberry and cranberry, and the respective genes are being cloned.

Ripe berry fruits are prone to fungal attack; hence it is conceivable that chitinases, proteases, and antifungal proteins are among the predominant allergens [13]. Strawberries are under investigation and strong evidence for the existing Bet v 6, Bet v 1 and Mal d 3 homologous proteins have been confirmed by existence of IgE-binding assays and skin prick test [68].

Several copies of PR-10 and PR-14 proteins were detected by Southern blots in strawberry, raspberry and black-berry fruit. In raspberry, the highest similarity at the DNA level for PR-10 and PR-14 (Rub i 1 and Rub i 3) was detected to strawberry sequences of Fra a 1 and Fra a 3. At the protein level, Rub i 1 and Rub i 3 showed more than 70% identity with homologous proteins of rosaceous fruits. Furthermore, raspberries contained additional putative allergens, e.g. class III acidic chitinases and cyclophilins. Blackberries were shown to share at least two well-known major fruit allergens with other rosaceous fruits, namely PR-10s and PR-14s homologous proteins. However the IgE-reactive proteins of small fruits are still not extensively investigated. The main challenges in studying small fruit allergens are the complexity of the fruit matrix, the diversity of physico-chemical properties of fruit proteins, the lack of appropriate protein extraction procedures and the missing information about the influence of processing treatments on food components.

Western blot experiments with polyclonal antibodies against known apple allergens demonstrated that a Mal d 1-homologous protein, related to birch pollen allergen Bet v 1 and belonging to PR 10 family [45], is also expressed in strawberry and blackberry protein extracts [74]. Likewise, the non-specific lipid transfer protein (LTP) Mal d 3, with antifungal and antibacterial activities and belonging to PR 14 family, is also expressed in raspberry, blueberry and blackberry fruit [36]. Strawberry Fra a 1 proteins consist of several IgE-binding peptides homologous to birch pollen Bet v 1 allergen [27, 43].

Proteomic studies demonstrated that Fra a 1 allergen level can be efficiently modulated by growth conditions (CV 43%); while its variation among genotypes is relatively low (CV 39%). Profilin and LTP allergens were also found in strawberry. Strawberry LTPs are accumulated at high levels in fruits and their synthesis is up-regulated by abiotic stress [73]. Other members of the LTP and profilin families were isolated from a strawberry fruit cDNA library and functionally characterized following heterologous expression in yeast. Strawberry LPTs might be used for immunotherapy, since they display a lower allergenicity than their apple or peach homologs [45]. Quite inter-

estingly, the absence of anthocyanin pigments in the white genotypes was associated with levels of Fra a 1 allergens significantly lower than in the red varieties, suggesting a sort of cross regulation between the allergen biosynthesis and anthocyanin pathways [22].

Allergic reactions were also reported in contact to raspberry proteins. Inhalation of frozen raspberry powder was reported to cause occupational asthma [23]. Quite recently Patiwael et al. [49] reported that strawberry greenhouse workers exhibited occupational allergy, concluding that allergic symptoms among a proportion of strawberry greenhouse employees were attributable to the workplace.

An initial analysis of genetic sequences within the Rosaceae indicates that coding regions of the most genes are conserved [17]. The high level of synteny among Rosaceous fruits like raspberry and apple, suggests a very similar protein expression pattern [3]. However, proteomic data on fruit proteins are difficult to obtain due to the complexity of the plant specific tissue matrix and the low protein content [22, 37].

2-DE in combination with IgE-Western blotting was applied for a selective allergen profiling in raspberries [21]. cDNA sequence analysis has already been shown to be a powerful tool in the characterisation of plant allergens [53]. In a recent study, four raspberry allergens belonging to three different protein classes were isolated: Rub i 1 and Rub i 3 are homologous to IgE-reactive Fra a 1 and Fra a 3 peptides, the other two are similar to "panallergenic" cyclophilin and class III chitinase peptides [38].

In raspberries, the proteomic approach in combination with PCR and MS/MS peptide sequencing revealed that the raspberry fruit shares at least two IgE-reactive proteins with other Rosaceous fruits, namely Rub i 1, a Mal d 1 homologous protein and Rub i 3, a Mal d 3 homologous nsLTP. However, the expression level of Rub i 1 seems to be very low suggesting a minor role in allergic reactions to raspberry. Additionally, two other proteins are likely to contribute to the allergenicity of raspberries, namely cyclophilin and class III chitinase, the later reacting with up to 80% of the tested sera. These results indicate that the IgE-reactivity of raspberry proteins is not only induced by known PR genes, but also by additional gene families. Though both cyclophilins and class III chitinase have already been identified as allergens in birch pollen and latex, respectively, more clinical investigations are necessary to understand the impact of cross-reactivity.

Despite the increasing information on berry structural flavonoid genes, only one Myb transcription factor (TF) has been characterized in strawberry, FaMyb 1. Overexpression of FaMyb 1 reduced anthocyanin and flavonol levels in flowers of transgenic tobacco lines, suggesting a repressor role in transcription [1].

A 2-year evaluation of different strawberry cultivars and breeding lines at different developmental stages demonstrated that ripe red fruits of the cultivar "Sveva", even though they contain a low content of anthocyanins, they showed the highest TAC, the most relevant TPC and TFC, and the poorest allergen content, thus distinguishing them from the other strawberry clones studied [4]. The content of the strawberry allergen Fra a 1 was determined by Dot Blot and the homologous protein Mal d 1 from apple was used for setting a standard curve [33]. The cultivar "Sveva" contained the lowest amounts of Fra a 1, whereas the highest levels of the allergen were present in the selection "AN94.414.52". These findings suggested that the controlled crosses of wild species with the cultivated strawberries may contribute to the introgression of interesting nutritional features, but also to the increase of unfavourable compounds, which might have health implications for humans [62]. Evaluation of the allergen concentrations during fruit ripening revealed an unusual accumulation pattern. For instance, only the "Adria" fruits showed a progressive decrease of the Fra a 1 content during fruit development; while in the other genotypes the developmental allergen concentrations fluctuated, suggesting a genetically determined response of allergen accumulation to the environmental factors [62].

Recently a proteomic approach was used to assess the differential protein expression in red and white strawberry cultivars, demonstrating a down-regulation of the Bet v 1 homologues allergen in white strawberries [22]. According to Musidlowska-Persson et al. [43] the sequence variability in the strawberry allergen Fra a 1 is small, within and between strawberry varieties, and that multiple spot previously detected in 2-DE, are presumably due to differences in post-translational modification rather than differences in amino acid sequence.

The strong correlation between the anthocyanin biosynthesis and Fra a 1 expression has been recently confirmed using a RNAi silencing strategy in strawberries, suggesting that the Fra a allergen has an essential biological function in pigment formation in the strawberry fruit [44]. In addition, the data from interspecific crosses showed that the wild species may contribute to the presence of interesting nutritional features in cultivated strawberries, but also to the increase of non-nutritional compounds, which might have implications for humans. Finally,

the results obtained stressed the importance of including the evaluation of genotype-environment interactions for assessing breeding programs. Only such a multidisciplinary approach will allow the creation of new varieties able to offer better quality and consumer safety, independently of the cultivation conditions and environmental changes.

Results obtained indicate that raspberries share a similar allergen pattern with apples. Rub i 1 and Rub i 3 show a sequence identity higher than 70% with homologous allergens in other Rosaceous fruits. In fact, proteins sharing more than 70% identity are commonly considered as cross-reactive [39]. The FAO/WHO expert panel recommendations even include an identity greater than 35% over 80 or more amino acids as a guideline to suggest possible cross-reactivity (FAO Corporate document repository; http://www.fao.org/docrep/003/x7133m/x7133m03.htm). However, the expression level of Rub i 1 in the analysed samples was too low to be visualised by Coomassie staining. An IgE-reactive protein spot of 18 kDa and a pI 9.0 was identified by MS/MS as a cyclophilin, with high amino acid homology to the birch pollen allergen Bet v 7. Therefore, the IgE-reactivity at approximately 18 kDa is caused by at least two proteins, namely Rub i 1 and a cyclophilin. Cyclophilins have been identified to catalyse peptidyl–prolyl isomerisation, during which the peptide bond preceding proline is stabilised in the cis-conformation, in all organisms studied from *Aspergillus fumigatus* and Mal s 6 from *M. sympodialis* [38]. An IgE binding cyclophilin from carrot is not cross-reactive with the birch pollen homologue [16], a feature which still needs to be investigated for the raspberry cyclophilin.

More significant was a triple protein band of ca. 30 kDa reacting with 80% of the tested patient sera. The MS analysis of each single protein spot confirmed all spots as class III chitinases carrying at least one N-glycan with a core a1,3 fucose and a b1,2 xylose, both known to be crucial for CCDs [38]. A class III chitinase from Indian jujube, Ziz m 1, has been reported recently as an allergen [32]. The protein sequence of Ziz m 1 indicates at least five possible glycosylation sites. Due to high sequence identity of class III chitinases and Hev b hevamine, a minor allergen from latex, the IgE-reactivity might be explained in the context of the latex-fruit syndrome [32]. However, reported allergies to banana and kiwi are caused by class I chitinase panallergens, which do not share any sequence similarity with class III chitinases. Class III chitinases lacking hevein domains are mainly of plant and fungal origin [32]. In the current study, the IgE-reactivity to class III chitinases was unexpected, since sera were obtained from patients with a predominant allergy to apples and raspberries, particularly to allergens like Mal d 1 and Mal d 3. Moreover the anamnesis contained only two cases of latex sensitivity, both of which were negative to Hev b 6.

In this study, 2-DE Western blots with polyclonal antibodies raised against the major apple allergens and sera from patients allergic to Rosaceous fruits, followed by MS/MS and LC-ESI-Q-TOF MS were applied to characterise IgE-reactive proteins from raspberry.

Blueberry fruits, although belonging to the *Ericaceae* family, share at least three IgE-reactive proteins with Rosaceous fruits, namely a Mal d 1 and a Mal d 3 homologous protein, as well as profilin. Quite recently, a clinical case of anaphylaxis was attributed to a 10 kDa lipid transfer protein: of blueberry [19]. However, more clinical investigations are necessary to understand the impact of blueberry allergens.

5. Conclusions

In the past decade, numerous efforts were undertaken to create genetic and proteomic identification tools for fruit allergens to obtain a fast evaluation of individual sensitisation patterns. Using these tools it was possible to discover in small fruits homologous genes belonging to the major known plant allergen families. Improved and more accurate analytics even allowed the discovery of novel allergens.

In an attempt to alleviate the situation of allergic patients, different technical approaches can be envisaged. On one hand, food processing, particularly thermal processing was observed to reduce the allergenicity of some foods, and therefore it was even proposed as contribution to a better management of the allergenic risk of foods [67]. A decrease or increase of allergenicity can be caused by protein unfolding, mis-folding or aggregation as well as by chemical modifications occurring during food processing by addition of sugar (e.g. Maillard reaction). For fruit manufacturer it is important to move toward a knowledge-based management of allergen risk assessment during fruit manufacturing [58].

Also breeding of cultivars low in allergens represents a viable, but long term alternative. Since avoidance of fruits was shown to negatively affect the quality of patients' lives, biotechnological interventions are ongoing to produce low allergenic fruits by down regulating specific genes. In this respect, the control of proteins associated with allergenicity could be achieved by fine tuning the spatial and temporal expression of the relevant genes.

References

[1] A. Aharoni, C.H.R. de Vos, M. Wein, Z. Sun, R. Greco, A. Kroon, J.N.M. Mol and A.P. O'Connell, The strawberry FaMYB1 transcription factor suppresses anthocyanin and flavonol accumulation in transgenic tobacco, *Plant J* **28** (2001), 319–332.

[2] R. Alm, A. Ekefjard, M. Krogh, J. Hakkinen and C. Emanuelsson, Proteomic variation is as large within as between strawberry varieties, *J Proteome Res* **6** (2007), 3011–3020.

[3] P. Arus, Integrationg genomics into Rosaceae fruit breeding, *Acta Hortic (ISHS)* **738** (2007), 29–35.

[4] M. Battino, J. Beekwilder, B. Denoyes-Rothan, M. Laimer, G.J. McDougall and B. Mezzetti, Bioactive compounds in berries relevant to human health, *Nutr Rev* **67** (2009), S145–S150.

[5] W.M. Becker and G. Reese, Immunological identification and characterization of individual food allergens, *J Chromatogr B Biomed Sci Appl* **756** (2001), 131–140.

[6] L. Berrens, A.G. van Dijk, G.F. Houben, M.L.D. Hagemans and W.J. Koers, Cross-reactivity among the pollen proteins and apple trees, *Allerg Immunol* **36** (1990), 147–156.

[7] F. Björksten, L. Halmepuro, M. Hannuksela and A. Lahti, Extraction and properties of apple allergens, *Allergy* **35** (1980), 671–677.

[8] H. Breiteneder, K. Pettenburger, A. Bito, R. Valenta, D. Kraft, H. Rumpold, et al., The gene coding for the major birch pollen allergen Bet v 1, is highly homologous to a pea disease resistance response gene, *EMBO J* **8** (1989), 1935–1938.

[9] H. Breiteneder and C. Ebner, Molecular and biochemical classification of plant-derived food allergens, *J Allergy Clin Immunol* **106** (2000), 27–36.

[10] H. Breiteneder and C. Radauer, A classification of plant food allergens, *J Allergy Clin Immunol* **113** (2004), 821–830.

[11] P. Cadot, J.F. Diaz, P. Proost, J. Van Damme, Y. Engelborghs, E.A. Stevens and J.L. Ceuppens, Purification and characterization of an 188 kd allergen of birch (*Betula verrucosa*) pollen: Identification as a cyclophilin, *J Allergy Clin Immunol* **105** (2000), 286–291.

[12] P. Cadot, L. Nelles, M. Srahna, E. Dilissen and J.L. Ceuppens, Cloning and expression of the cyclophilin Bet v 7, and analysis of immunological cross 8 reactivity among the cyclophilin A family, *Mol Immunol* **43** (2006), 226–235.

[13] F. Carbone, F. Mourgues, G. Perrotta and C. Rosati, Advances in functional research of antioxidants and organoleptic traits in berry crops, *BioFactors* **34** (2008), 23–36.

[14] M. Fernandez-Rivas, R. van Ree and M. Cuevas, Allergy to Rosaceae fruits without related pollinosis, *J Allergy Clin Immunol* **100** (1997), 728–733.

[15] M. Fernandez-Rivas and M. Cuevas, Peels of Rosaceae fruits have a higher allergenicity than pulps, *Clin Exp Allergy* **29** (1999), 1239–1247.

[16] C. Fujita, T. Moriyama and T. Ogawa, Identification of cyclophilin as an IgE8 binding protein from carrots, *Int Arch Allergy Immunol* **125** (2001), 44–50.

[17] M.D. Gale and K.M. Devos, Plant comparative genetics after 10 years, *Science* **282** (1998), 656–659.

[18] M. Gavrović-Jankulović, M. Spasić, T. Cirković Velicković, M. Stojanović, A. Inić-Kanada, L. Dimitrijević, B. Lindner, A. Petersen, W. Becker and R. Jankov, Quantification of the thaumatin-like kiwi allergen by a monoclonal antibody-based ELISA, *Mol Nutr Food Res* **52** (2008), 701–707.

[19] C. Gebhardt, S. Vieths, M. Gubesch, M. Averbeck, J.C. Simon and R. Treudler, 10 kDa lipid transfer protein: The main allergenic structure in a German patient with anaphylaxis to blueberry, *Allergy* **64** (2009), 498–499.

[20] R.M. Helm and A.W. Burks, Mechanisms of food allergy, *Curr Opin Immunol* **12** (2000), 647–653.

[21] A. Herndl, G. Marzban, D. Kolarich, R. Hahn, D. Boscia, W. Hemmer, F. Maghuly, E. Stoyanova, H. Katinger and M. Laimer, Mapping of *Malus domestica* allergens by two-dimensional electrophoresis and IgE-reactivity, *Electrophoresis* **28** (2007), 437–448.

[22] K. Hjernø, R. Alm, B. Canbäck, R. Matthiesen, K. Trajkovski, L. Björk, P. Roespstorff and C. Emanuelsson, Down-regulation of the strawberry Bet v 1-homologous allergen in concert with the flavonoid biosynthesis pathway in colorlessstrawberry mutant, *Proteomics* **6** (2006), 1574–1587.

[23] K. Hoffmann-Sommergruber, Pathogenesis-related (PR)-proteins identified as allergens, *Biochem Soc Trans* **30** (2002), 930–935.

[24] L. Hsieh, M. Moos and Y. Lin, Characterization of apple 18 and 31 kd allergens by microsequencing and evaluation of their content during storage and ripening, *J Allergy Clin Immunol* **96** (1995), 960–970.

[25] L. Jaeger and B. Wuethrich, Nahrungsmittelallergien und -intoleranzen: Immunologie, Diagnostik, Therapie und Prophylaxe, Fischer, Ulm, 1998.

[26] J. Kader, Lipid transfer proteins in plants, *Annu Rev Plant Physiol* **47** (1996), 627–654.

[27] A.L. Karlsson, R. Alm, B. Ekstrand, S. Fjelkner-Modig, A. Schiött, U. Bengtsson, L. Börk, K. Hjernø, P. Roepstorff and C.S. Emanuelsson, Bet v 1 homologues in strawberry identified as IgE-binding proteins and presumptive allergens, *Allergy* **59** (2004), 1277–1284.

[28] A. Kasprzewska, Plant chitinases – regulation and function, *Cell Mol Biol Lett* **8** (2003), 809–824.

[29] T.P. King, D. Hoffman, H. Löwenstein, D.G. Marsh, T.A.E. Platts-Mills and W. Thomas, Allergen nomenclature, *Allergy* **50** (1995), 765–774.

[30] E. Knapp, A. da Câmara Machado, H. Pühringer, Q. Wang, V. Hanzer, H. Weiss, B. Weiss, H. Katinger and M. Laimer da Câmara, Localization of fruit tree viruses by immuno-tissue printing in infected shoots of *Malus* and *Prunus* sp., *J Virol Meth* **55** (1995), 157–173.

[31] M. Krebitz, B. Wagner, F. Ferreira, C. Peterbauer, N. Campillo, M. Witty, et al., Plant-based heterologous expression of Mal d 2, a thaumatin-like protein and allergen of apple (*Malus domestica*), and its characterization as an antifungal protein, *J Mol Biol* **329** (2003), 721–730.

[32] M.-F. Lee, Y.-H. Chen, H.-C. Lin, H.-L. Wang, G.-Y. Hwang and C.-H. Wu, Identification of hevamine and hev B 1 as major latex allergens in Taiwan, *Int Arch Allergy Immunol* **139** (2006), 38–44.

[33] F. Maghuly, G. Marzban and M. Laimer, Functional genomics of allergen gene families in fruits, *Nutrients* **1** (2009), 119–132.

[34] Z. Markovic-Housley, M. Degano, D. Lamba, E. von Roepenack-Lahaye, S. Clemens, M. Susani, et al., Crystal structure of a hypoallergenic isoform of the major birch pollen allergen Bet v 1 and its likely biological function as a plant steroid carrier, *J Mol Biol* **325** (2003), 123–133.

[35] G. Marzban, H. Pühringer, R. Dey, S. Brynda, A. Martinelli, M. Zaccarini, D. Kolarich, F. Altmann, H. Katinger and M. Laimer, Localisation and distribution of major apple allergens in fruit tissue, *Plant Sci* **169** (2005), 387–394.

[36] G. Marzban, A. Mansfeld, W. Hemmer, E. Stoyanova, H. Katinger and M.L. da Camara Machado, Fruit cross-reactive allergens: A theme of uprising interest for consumers' health, *BioFactors* **23** (2005), 235–241.

[37] G. Marzban, A. Mansfeld, A. Herndl, S. Jäger, M.E. Stoyanova, W. Hemmer, H. Katinger and M. Laimer, Direct evidence for the presence of allergens in *Rosaceae* fruit-tree pollen, *Aerobiologia* **22** (2006), 237–245.

[38] G. Marzban, A. Herndl, D. Kolarich, F. Maghuly, A. Mansfeld, W. Hemmer, H. Katinger and M. Laimer, Identification of four IgE-reactive proteins in raspberry (*Rubus ideaeus* L.), *Mol Nutr Food Res* **52** (2008),1497–1506.

[39] G. Marzban, F. Maghuly, A. Herndl, H. Katinger and M. Laimer, Screening and identification of putative allergens in berry fruits of the *Rosaceae* family: Technical challenges, *BioFactors* **33** (2008), 1–10.

[40] G. Marzban, A. Herndl, S. Pietrozotto, S. Banerjee, C. Obinger, F. Maghuly, R. Hahn, D. Boscia, H. Katinger and M. Laimer, Conformational changes of Mal d 2, a thaumatin-like apple allergen, induced by food processing, *Food Chem* **112** (2009), 803–811.

[41] J.E. Mogensen, R. Wimmer, J.N. Larsen, M.D. Spangfort and D.E. Otzen, The major birch allergen, Bet v 1, shows affinity for a broad spectrum of physiological ligands, *J Biol Chem* **277** (2002), 23684–23692.

[42] A. Molina, I. Diaz, I. K. Vasil, P. Carbonero and F. Garcia-Olmedo, Two cold-inducible genes encoding lipid transfer protein LTP4 from barley show differential responses to bacterial pathogens, *Mol Gen Genet* **252** (1996), 162–168.

[43] A. Musidlowska-Persson, R. Alm and C. Emanuelsson, Cloning and sequencing of the Bet v 1-homologous allergen Fraa1in strawberry (*Fragaria ananassa*) shows the presence of an intron and little variability in amino acid sequence, *Mol Immunol* **44** (2007), 1245–1252.

[44] C. Muñoz, T. Hoffmann, N. Medina Escobar, F. Ludemann, M.A. Botella, V. Valpuesta and W. Schwab, The strawberry fruit Fra a allergen functions in flavonoid biosynthesis, *Mol Plant* (2009). http://dx.doi.org/10.1093/mp/ssp087

[45] P. Neudecker, K. Schweimer, J. Nerkamp, S. Scheurer, S. Vieths, H. Sticht, et al., Allergic cross-reactivity made visible: Solution structure of the major cherry allergen Pru av 1, *J Biol Chem* **276** (2001), 22756–22763.

[46] E.A. Pastorello, L. Farioli, V. Pravettoni, C. Ortolani, M. Ispano, M. Monza, C. Baroglio, E. Scibola, R. Ansaloni, C. Incorvaia and A. Conti, The major allergen of peach (*Prunus persica*) is a lipid transfer protein, *J Allergy Clin Immunol* **103** (1999), 520–526.

[47] E.A. Pastorello, F.P. D'Ambrosio, V. Pravettoni, L. Farioli, G. Giuffrida, M. Monza, R. Ansaloni, D. Fortunato, E. Scibola, F. Rivolta, C. Incorvaia, A. Bengtsson, A. Conti and C. Ortolani, Evidence for a lipid transfer protein as the major allergen of apricot, *J Allergy Clin Immunol* **105** (2000), 371–377.

[48] E.A. Pastorello and C. Trambaioli, Isolation of food allergens, *J Chromatogr B Biomed Sci Appl* **756** (2001), 71–84.

[49] J.A. Patiwael, L.G.J. Vullings, N.W. de Jong, A.W. van Toorenenbergen, R. Gerth van Wijk and H. de Groot, Occupational allergy in strawberry greenhouse workers, *Int Arch Allergy Immunol* **152** (2010), 58–65.

[50] R.E. Poms, C.L. Klein and E. Anklam, Methods for allergen analysis in food: A review, *Food Addit Contam* **21** (2004), 1–31.

[51] L.K. Poulsen, *In vivo* and *in vitro* techniques to determine the biological activity of food allergens, *J Chromatogr B Biomed Sci Appl* **756** (2001), 41–55.

[52] H. Pühringer, D. Moll, K. Hoffmann-Sommergruber, B. Watillon, H. Katinger and M. Laimer da Câmara Machado, The promoter of an apple YPR10 gene, encoding the major apple allergen Mal d 1, is stress and pathogen-inducible, *Plant Sci* **152** (2000), 35–50.

[53] H. Pühringer, I. Zinoecker, G. Marzban, H. Katinger and M. Laimer, MdAP, a novel protein in apple, is associated with the major allergen Mal d 1, *Gene* **321** (2003), 173–183.

[54] C. Radauer and K. Hoffmann-Sommergruber, Profilins. in: *Plant Food Allergens*, E.N.C. Mills and P.R. Shewry, eds, Blackwell Publishing, London, 2004, pp. 105–124.

[55] S. Ramachandran, H.E. Christensen, Y. Ishimaru, C.H. Dong, W. Chao-Ming, A.L. Cleary, et al., Profilin plays a role in cell elongation, cell shape maintenance, and flowering in *Arabidopsis*, *Plant Physiol* **124** (2000), 1637–1647.

[56] P. Restani, C. Ballabio, A. Cattaneo, P. Isoardi, L. Terracciano and A. Fiocchi, Characterization of bovine serum albumin epitopes and their role in allergic reactions, *Allergy* **59**(Suppl 78) (2004), 21–24.

[57] W. Roberts and C. Selitrennikoff, Zeamatin, an antifungal protein from maize with membrane-permeabilizing activity, *J Gen Microbiol* **136** (1990), 1771–1778.

[58] A.I. Sancho, R. Foxall, N.M. Rigby, T. Browne, L. Zuidmeer, R. van Ree, K.W. Waldron and C. Mills, Maturity and storage influence on the apple (*Malus domestica*) allergen Mal d 3, a nonspecific lipid transfer protein, *J Agric Food Chem* **54** (2006), 5098–5104.

[59] U. Smole, M. Bublin, C. Radauer, C. Ebner and H. Breiteneder, Mal d 2, the thaumatin-like allergen from apple, is highly resistant to gastrointestinal digestion and thermal processing, *Int Arch Allergy Immunol* **147** (2008), 289–298.

[60] I. Somssich, E. Schmelzer, J. Bollmann and K. Hahlbrock, Rapid activation by fungal elicitor of genes encoding 'pathogenesis-related' proteins in cultured parsley cells, *Proc Natl Acad Sci USA* **83** (1986), 2427–2430.

[61] V. Tumbas, J. Čanadanović-Brunet, L. Gille, S. Đilas and G. Ćetković, Superoxide anion radical scavenging activity of bilberry (*Vaccinium myrtillus* L.), *J Berry Res* **1** (2010), 13–23.

[62] S. Tulipani, G. Marzban, A. Herndl, M. Laimer, B. Mezzetti and M. Battino, Influence of environmental and genetic factors on health-related compounds in strawberry, *Food Chem* **124** (2011), 906–913.

[63] L.C. van Loon and E.A. Van Strien, The families of pathogenesis related proteins, their activities, and comparative analysis of PR-1-type proteins, *Physiol Mol Plant Pathol* **5** (1999), 85–97.

[64] R. van Ree, Clinical importance of non-specific lipid transfer proteins as food allergens, *Biochem Soc Trans* **30** (2002), 910–913.

[65] A.H. Valster, E.S. Pierson, R. Valenta, P.K. Hepler and A.M.C. Emons, Probing the plant actin cytoskeleton during cytokinesis and interphase by profilin microinjection, *Plant Cell* **9** (1997), 1815–1824.

[66] S. Vieths, A. Jankiewicz, B. Schöning and H. Aulepp, The IgE binding potency of apple strains is related to the occurrence of the 18-kDa apple allergen, *Allergy* **49** (1994), 262–271.

[67] J.-M. Wal, Thermal processing and allergenicity of foods, *Allergy* **58** (2003), 727–729.

[68] M. Walter, J. Liu, C. Grand, C. Lamb and D. Hess, Pathogenesis-related (PR) proteins deduced from elicitor-induced transcripts are members of a ubiquitous new class of conserved PR proteins including pollen allergens, *Mol Gen Genet* **222** (1990), 353–360.

[69] M. Walter, J. Liu, J. Wunn and D. Hess, Bean ribonuclease-like pathogenesis-related protein genes (Ypr10) display complex patterns of developmental, dark-induced and exogenous-stimulus-dependent expression, *Eur J Biochem* **239** (1996), 281–293.

[70] P. Wang and J. Heitman, The cyclophilins, *Genome Biol* **6** (2005), 226.

[71] W. Weiss and A. Gorg, Two-dimensional electrophoresis for plant proteomics, *Methods Mol Biol* **355** (2007), 121–143.

[72] M. Wensing, J.H. Akkerdaas, W.A. van Leeuwen, S.O. Stapel, C.A. Bruijnzeel-Koomen, R.C. Aalberse, et al., IgE to Bet v 1 and profilin: Cross-reactivity patterns and clinical relevance, *J Allergy Clin Immunol* **110** (2002), 435–442.

[73] E.M. Yubero-Serrano, E. Moyano, N. Medina-Escobar, J. Muñoz-Blanco and J.L. Caballero, Identification of a strawberry gene encoding a non-specific lipid transfer protein that responds to ABA, wounding and cold stress, *J Exp Bot* **54** (2003), 1865–1877.

[74] L. Zuidmeer, W.A.I.K. van Leeuwen, Budde, J. Cornelissen, I. Bulder, I. Rafalska, N.T. Besoli, J.H. Akkerdaas, R. Asero, M. Fernandez Rivas, E. Gonzalez Mancebo and R. van Ree, Lipid transfer proteins from fruit: Cloning, expression and quantification, *Int Arch Allergy Immunol* **137** (2005), 273–281.