

Localisation and distribution of the major allergens in apple fruits

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Abstract

The importance of apple allergens, in particular Mal d 1, a Bet v 1 homologue for the pollen-fruit syndrome in Northern Europe, and Mal d 3, responsible for true fruit allergy in Southern Europe, has been repeatedly emphasized. However, little is known about the distribution pattern of major allergens in fruits and whether differences exist among different cultivars. Transcript expression of Mal d 1 isoforms and Mal d 3 was examined by RealTime-PCR and Northern analysis, respectively. An immuno-tissue-print (ITP) assay was developed to localise major allergens in apple fruit tissue and a Mal d 1 specific, patient independent ELISA was established. ITP analyses show that Mal d 1 and Mal d 2 are distributed throughout the apple pulp and peel, while Mal d 3 is restricted to the peel. Data obtained by ELISA reveal a variation of Mal d 1 content ranging from 0.84 to 33.17 µg/g fresh weight in 39 selected cultivars. Different apple cultivars show a markedly different expression of major allergens; this finding will influence the development of diagnostic tools as well as the dietary management of allergic individuals.

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1. Introduction

Apple is the most widely grown and consumed fruit in Europe (a total of 10.04 million metric tons in the year 2002). Currently the European Union is actively encouraging consumption of fruits and vegetables for positive health standards. Although it is well known that increased intake of apples and other vegetables can reduce the risk of chronic diseases such as heart disease, strokes and some cancers, it may also impose an allergenic risk [1,2]. On the basis of several epidemiological studies at national levels it can be

calculated that at least two millions EU citizens are affected by various fruit allergies [3].

In Central and Northern Europe as well as in Northern America up to 90% of patients with birch pollen allergy develop intolerance to fruits and/or vegetables [4]. Proteins with high homology in the primary sequence display also homologous tertiary structures, resulting in similar epitopes to IgEs and cross-reactivity. In apple, the 17.5 kDa protein Mal d 1 is the major allergen in pollen-associated fruit allergies, showing high sequence homology to the major birch pollen allergen Bet v 1 [5]. At least two further proteins are involved in the birch-apple syndrome: Mal d 2, a 31 kDa thaumatin-like protein with anti-fungal activity [6,7] and Mal d 4, a 14 kDa a proline-binding protein, well known as the pan-allergen profilin [5].

In Mediterranean countries, where basically no birches are growing, allergic reactions to fruits are predominantly

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evoked by non-specific lipid transfer proteins (nsLTPs). Interestingly, Mal d 3 – a 9 kDa nsLTP in apple – cross-reacts with homologous proteins in Rosaceous fruits, like pear, peach and apricot [3,8] as well as with LTPs from a number of non-Rosaceae vegetable food [9,10]. The frequency and severity of allergic reactions observed in different geographic regions is correlated with the route of sensitisation and the stability of the sensitising agent. Proteinase-sensitive allergens involved in the Bet v 1 ‘primed’ fruit allergy mostly cause mild symptoms such as the so-called oral allergy syndrome (OAS). In contrast, the highly heat and pepsin resistant nsLTPs survive the gastrointestinal tract and frequently provoke more severe symptoms, including urticaria, asthma, vomiting, diarrhoea, hypotension and even anaphylactic shock [11].

The availability of reliable *in vivo* and *in vitro* diagnostics for fruit allergy testing has frequently been limited by broad variations in commercial allergen preparations concerning purity, stability and concentration. In order to evaluate the individual expression levels of apple allergens known so far, relevant screening assays are prerequisite. In the present paper we describe the development of tools and the assessment of concentration and distribution of the major apple allergens in a large number of apple cultivars by several sensitive, patient-independent assays.

2. Methods

2.1. cDNA cloning and generation of DIG-labelled riboprobes for Mal d 1 and Mal d 3

All DNA manipulation and cloning techniques were performed as already described [12]. 1.2 µg total RNA of four cultivars (Granny Smith, Fuji, Jonagold and VistaBella) was reverse transcribed and used as template to amplify the coding region of Mal d 3 by polymerase chain reaction (PCR) using the degenerated primers LTP-F 5′ ATG GCT WGC TCT GCA RTG AYYAAG 3′ and LTP-R 5′ TYA CTT CAC GGT GGC GCA GTT G 3′. The 25 µl amplification reaction consisting of 50 mM KCl, 10 mM Tris pH 9.0, 0.1% Triton X-100, 2.5 mM MgCl₂, 200 µM dNTPs, 200 nM primers each and 0.5 U Taq polymerase (Promega, USA) was subjected to the following cycling conditions: initial denaturation at 94 °C 3 min, 35 cycles of 94 °C 45 s, 64 °C 1 min, 72 °C 45 s, and a final extension at 72 °C for 5 min. The 348 bp amplicon was ligated into the TA cloning vector pCRII (Invitrogen, The Netherlands) and transformed in *E. coli* INVαF⁺. Inserts from five individual clones per cultivar were sequenced with vector specific primers. The linearised and purified plasmid was transcribed *in vitro* by the SP6 RNA polymerase using the DIG RNA labelling mix (Roche, Switzerland).

The antisense riboprobe Mal d 1 was transcribed *in vitro* from the Mal d 1 coding region [13] cloned into pCRII (Stratagene, USA) using the DIG RNA labeling kit (Roche).

2.2. Northern blot analysis and quantification of Mal d 1 isoform transcripts

Total RNA was extracted from apple peel or pulp according to the method of Chang et al. [14]. Gel electrophoresis, blotting, hybridisation and detection of DIG-labelled probes were carried out as reported [13,15]. Quantitative expression of four Mal d 1 isoforms was measured by means of SYBR[®]Green I based RealTime-PCR. cDNA synthesis and RealTime-PCR experiments were performed as described previously [16].

2.3. Apple material and protein extraction

Ripe apples of commercially available cultivars, purchased from the supermarket, were washed extensively with hot water before preparation. Peel and pulp were extracted separately. RealTime-PCR experiments were performed to peel and pulp of same ratio per cultivar. Apple material was homogenised to fine powder under liquid nitrogen in a mortar.

For cultivar screening apples from an experimental field on sandy soils near the Adriatic seaside with drip irrigation systems were harvested at cultivar specific dates, and stored for 3 months at 4 °C before extraction. For the conventional samples integrated plant protection was carried out according to Reg. CEE 2078, including herbicide treatment within the rows. Organic production in these experiments means the mechanical elimination of weeds within the rows, and chemical control against insects according to Reg. CEE 2078, but no anti-fungal treatment. To assure a valid sampling, five apples per cultivar were peeled (5 mm) with a paring machine, shock frozen and powdered in liquid nitrogen. Frozen powder was mixed in a blender with extraction buffer (10 mM K₂HPO₄, 10 mM KH₂PO₄, 0.27 mM EDTA, 13.3 mM DIECA, 2% PVPP, pH7) in a ratio of 1:2 w/v and extracted by stirring at +4 °C in the presence of a protease inhibitor cocktail (Roche, Germany). The mixture was filtered through two layers of Miracloth (Calbiochem, USA), centrifuged at 10,000 × g for 30 min at +4 °C and the supernatant immediately frozen at –80 °C. Concentration of total protein was carried using BCA protein assay kit (PIERCE, USA) according to manufacturer’s instructions. All extracts were buffer-exchanged using PD-10 columns (Amersham Biotech, USA) before protein determination using BCA due to high contents of reducing agents in apple tissue.

2.4. Production of antibodies directed to apple allergens

Purified Mal d 1 used for the production of pAbs and mAbs, as well as for the standardisation of ELISA readings, were qualified using mass spectrometry. Mal d 1 gave ions of the charge states +12 to +19 under the conditions described. Deconvolution of these spectra with MaxEnt1 yielded a mass of 17407 ± 1.4 Da, which can be correlated

to the available sequence data, whereas one amino acid might be missing. Polyclonal antiserum against Mal d 1 was raised in rabbit by immunisation with 250 µg recombinant Mal d 1 (identical to Mal d 1a isolated from Golden Delicious Acc. No. AF126402) and Freund's adjuvants. The animal was boosted twice at two weeks intervals with 125 µg Mal d 1 and bled two weeks after the last boost. The monoclonal antibodies (6B9, 3H8A4, 1E10G9, 4C3G9, and 4C3C10) were obtained by hybridoma technology after repeated immunisation of mice with purified Mal d 1 (kindly provided by L. Zuidmeer), the latter is used for establishment of ELISA and other immunological assays. Polyclonal antibodies directed to Mal d 2 and peach LTP (cross-reactive with Mal d 3) were kindly provided by R. van Ree.

2.5. Immuno-tissue-prints (ITP)

To localise allergens in the apple fruit an ITP-assay was established. 0.2 µm Nitrocellulose membranes (Biorad, USA) were soaked in 100mM ascorbic acid (to prevent oxidation) and air dried prior to printing. ~1 mm slices of apple fruits were placed on pre-treated membranes. Tissue sections were covered with filter paper and gently pressed onto the membrane using a glass plate [17]. The membrane was air-dried and stored at room temperature (RT). Prints were incubated in blocking buffer in TBS (10 mM Tris-HCl pH 7.4, 150 mM NaCl) + 0.05% Tween20 + 5% defatted dried milk for 2 h, briefly rinsed in washing buffer (TBS + 0.05% Tween20) and incubated for 1 h with polyclonal rabbit antibodies, diluted 1:1000 in dilution buffer (TBS + 0.05% Tween20 + 1% defatted milk + 0.5% BSA). After 3 washes (10 min each) ITPs were incubated for 1 h in alkaline phosphatase labelled goat anti-rabbit IgG (Sigma A3687) diluted 1:5000 in dilution buffer. Washing was carried out as above with a final wash of 5 min in substrate buffer (100 mM Tris-HCl, pH 9.5 + 100 mM NaCl + 5 mM MgCl₂). All steps were carried out at room temperature with gentle agitation. Membranes were developed in 15 ml substrate buffer supplemented with 50 µl 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (50 mg/ml) + 50 µl nitroblue tetrazolium (NBT) (100 mg/ml) for 15 min in the dark (without shaking), briefly rinsed with tap water and air-dried.

2.6. Western blotting

Western blot was performed in a Novex-Gel apparatus and used precasted 4–20% Tris/Glycine acrylamide gels (Invitrogen, The Netherlands). After separation the proteins were transferred by electroblotting to a PVDF (polyvinylidene difluoride) membrane (Millipore, USA) and using as first antibody a polyclonal rabbit anti-Mal d 1 or the mouse monoclonal Ab 4C3C10 and as second antibody a anti-IgG mouse/rabbit labelled with alkaline phosphatase (Sigma, USA). As molecular weight marker pre-stained Precision Plus Protein Standards (Biorad, USA) was used.

To determine the specificity of the pAbs and mAbs developed for the patient independent ELISA, a comparison with radio labelled human sera was carried out in Western blots. The blotted proteins were incubated with allergic patients' sera (1:5) overnight at 4 °C and detected by 1:20 diluted ¹²⁵I labelled rabbit anti-human IgE (Med-Pro, Austria). Autoradiography was performed at room temperature for 12–48 h with intensifying phosphor screens and imaged on the storm 860 Imager (Amersham Biosciences, Sweden).

2.7. Enzyme-linked immuno sorbent assay (ELISA)

Microtiter plates (Nunc-Immuno Plate MaxiSorp™ Surface) were coated with 100 µl/well polyclonal rabbit anti-Mal d 1 antibody diluted 1:1000 in coating buffer (8.4 g/l NaHCO₃ + 4.0 g/l Na₂CO₃, pH 9.6) at +4 °C over night. The following consecutive incubation steps (100 µl/well) with three washes (300 µl TBS + 0.05% Tween20) between each step were: (1) apple extracts diluted 1:5 to 1:40 in dual steps (37 °C and 1 h) and purified native Mal d 1 as reference (500–7.8 ng/ml); (2) mouse mAb 4C3C10 1:500 (37 °C and 1 h); (3) alkaline phosphatase labelled goat anti-mouse IgG (Fab specific; Sigma A1682) 1:2000 (37 °C and 1 h); (4) *p*-nitrophenylphosphate (1 mg/ml) in coating buffer supplemented with 5 mM MgCl₂ (at room temperature and 90 min in the dark).

Samples and antibodies were diluted in TBS + 2% PVP + 0.5% BSA + 0.1% Tween20. Photometric measurement was performed at 405 nm (reference wavelength 620 nm) with a microplate reader. Absorbance values were expressed as ng Mal d 1 per ml extract by interpolation with the reference curve (DeltaSoft from BioMetallics). All determinations were run in duplicates and Mal d 1 amounts were converted to microgram Mal d 1 per gram fresh weight.

3. Results

3.1. Allergen expression in apple peel and pulp

Four apple cultivars analysed express the major allergens, Mal d 1 and Mal d 3. Both allergens were detected by Northern analysis as single bands of ~800 nucleotides (Mal d 1) and of ~650 nt (Mal d 3), respectively (Fig. 1). Mal d 1 transcripts were expressed in peel and pulp. In contrast, the Mal d 3 transcripts were found in apple peel samples, while no expression could be measured in pulp tissue. Cloned cDNA sequences of Mal d 3 from two different cultivars, Granny Smith and Golden Delicious (Acc. No. AF221502) show almost identical coding regions, except for three mismatches resulting in two amino acid exchanges in the 5' region. No cultivar-specific sequences were identified. Golden Delicious and Jonagold, as well as Granny Smith (Acc. No. AY374225), Fuji and VistaBella display identical Mal d 3 sequence. However, as the amino acid substitutions match the positions

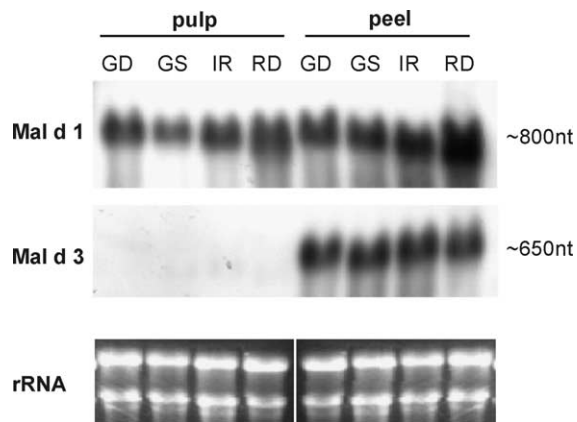


Fig. 1. Northern analysis of allergen transcript expression in apple fruit tissue. Five micrograms total RNA separated on a 1.5% denaturing agarose gel was blotted to a nylon membrane and hybridised to the respective DIG-labelled antisense-riboprobe under high stringent conditions. Chemiluminescent signals were recorded on X-ray film after 30 min exposure. Equal loading of the gel was checked by EtBr stained rRNA (lower panel). Approximate transcript size is given in nucleotides on the right side. GD = Golden Delicious, GS = Granny Smith, IR = Idared, RD = Red Delicious.

in the degenerated forward primer the sequence divergency could also result from amplification and not from a true polymorphism. The alignment of nsLTPs from *Rosaceae* fruits – described as major allergens [10,18,19] – displays a high degree of sequence similarity and the highly conserved position of nine cysteine residues [20].

3.2. Localisation of apple allergens in fruits

An ITP was established for Mal d 1, Mal d 2 and Mal d 3, respectively. Results of localisation studies (Fig. 2) show Mal d 1 and Mal d 2 equally distributed throughout the pulp in all cultivars. In all investigated cultivars Mal d 3 was absent in the pulp, but clearly detectable in the peel and seemed to be restricted to it. The observed distribution pattern match well to the Northern blot data.

3.3. Polymorphism in Mal d 1 transcript expression in apple fruits

Mal d 1 mRNAs, encoded by a multigene family, are transcribed in a similar size, however, a considerable diversity in their deduced amino acid sequence has been demonstrated [13,21,22]. Recently, the numerous sequences encoding Mal d 1 were clustered into four groups on the basis of their sequence similarity in the coding region: Mal d 1.01–Mal d 1.04 [16]. The expression profile of the four major isoform clusters was investigated in the whole fruits (Fig. 3) of unrelated cultivars. A clear pattern concerning the prevalence of isoform clusters could be observed in all tested cultivars. Mal d 1.02 (Acc. No. AF020542) was by far the most abundantly expressed isoform in ripe apple, followed by an almost ten times lower expression of Mal d 1.01 (Acc. No. AJ417551) and a significantly lower expression of Mal d 1.03 (Acc. No. AY186248). In none of the tested samples

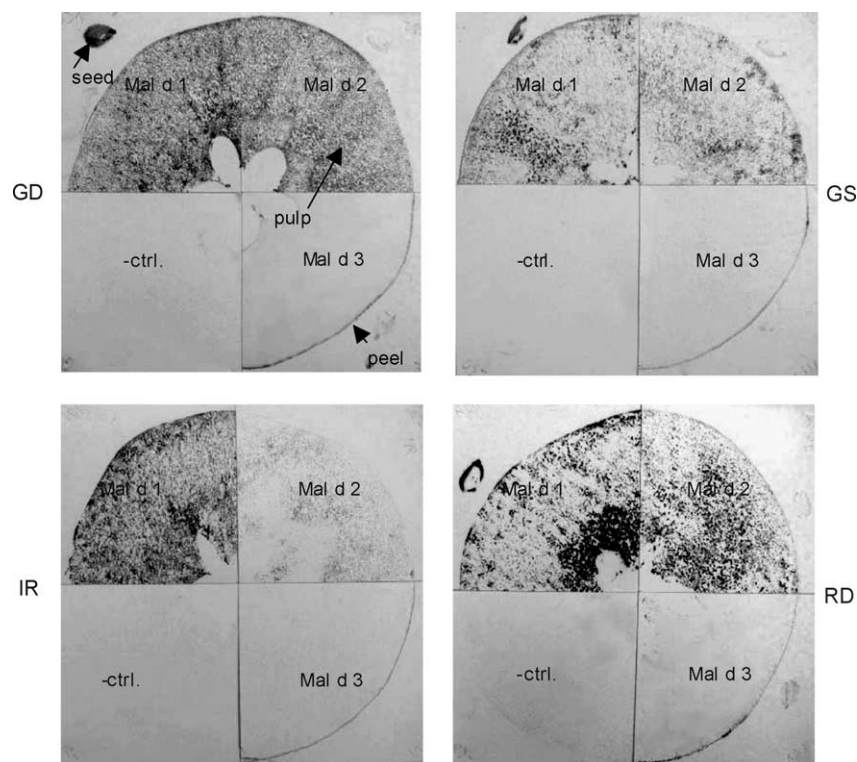


Fig. 2. Allergen distribution in apple fruits investigated by ITP. Imprints of apple fruit slices (vertical to core axis) were cut into four sectors and incubated with the respective antibody specific for the major allergens Mal d 1, Mal d 2 and Mal d 3. –ctrl = negative control was incubated with conjugated second step antibody only. GD = Golden Delicious, GS = Granny Smith, IR = Idared, RD = Red Delicious.

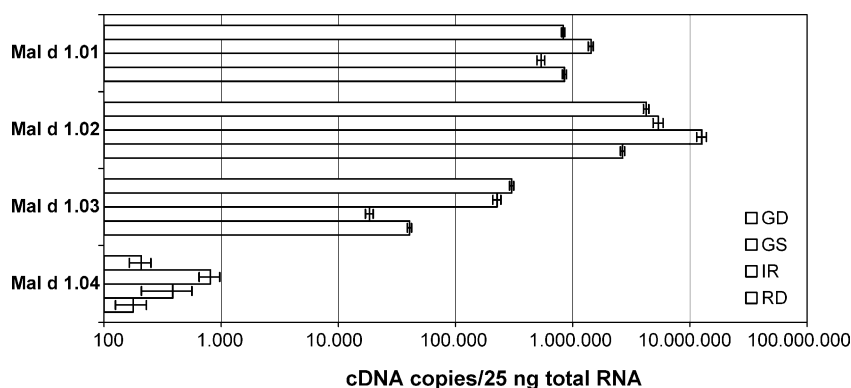


Fig. 3. Quantification of Mal d 1 isoform transcripts in apple by RealTime-Q PCR. Results are expressed as cDNA copies per 25 ng total RNA extracted from the same ratio of pulp and peel per cultivar. Values represent the mean $\pm$ S.D. of at least two independent measurements each with triplicates. GD = Golden Delicious, GS = Granny Smith, IR = Idared, RD = Red Delicious.

Mal d 1.04 (Acc. No. [Z72428](#)) transcripts could be measured unequivocally, as the expression level was below the reliable detection limit of the applied assay. These data, from four unrelated cultivars, match to data obtained recently from the two related cultivars Golden Delicious and Jonagold [16] and from different tissues [23].

3.4. Specificity of mAb and pAb for Mal d 1 in apple extract

The specificity of the pAb and the mAbs used in the patient independent ELISA was determined by Western blot. Three apple extracts (low to medium, and high in ELISA) were detected with the pAb, the mAbs, BIP (anti Bet v 1 mAb) and two different Austrian patient sera (Fig. 4). Interestingly, all sera recognised the major band at ca. 17 kDa with an intensity corresponding to the respective ELISA data. Two-dimensional western blotting of apple extract has shown that at least four isoforms can be detected by mAb and pAb (Marzban et al., in prep.).

3.5. Mal d 1 expression varies within apples and between cultivars

The distribution and content of Mal d 1 of cultivars purchased from supermarket were determined. For quantification four pulp samples per apple from five apples per

cultivar were analysed, yielding highly variable Mal d 1 contents between individual apples (Fig. 5A). Golden Delicious and Jonagold showed significant differences within individual apples, whereas Gala and Elstar had a lower inter-apple variability. Intra-apple variability was particularly high in Jonagold (Fig. 5B), and low in Gala and Elstar (Fig. 5A). Considering the small SD bars in Fig. 5B deviating values were clearly due to different Mal d 1 amount in the samples and not due to assay conditions. Preliminary investigations of Mal d 1 distribution pattern in peel and pulp from individual apples indicate a random accumulation (Fig. 5C). Thirty-nine apple cultivars, including currently commercialized, locally grown old cultivars and new breeding lines, which still did not enter the market place were chosen and screened for their Mal d 1 content ($\mu\text{g/g}$ fresh weight). Total protein determination by BCA assay yielded accurate data on protein content in cultivar extracts, ranging from 1.07 to 4.69 mg/g apple fresh weight, which is in accordance with data reported in the literature [24]. Mal d 1 content was correlated to total protein content and expressed in % Mal d 1/total soluble protein. Cultivars could be classified into three groups: low (below 5 $\mu\text{g/g}$), medium (5–10 $\mu\text{g/g}$) and high (>10 $\mu\text{g/g}$) (Table 1). Cultivar ranking by Mal d 1 content showed significant similarity to ranking based on % Mal d 1/total soluble protein. A hierarchical cluster analysis of these two parameters with average linkage confirmed the three well-

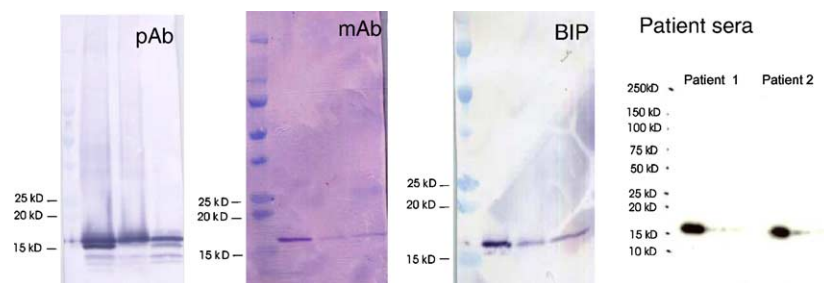


Fig. 4. Western blot is used to qualify mAbs used for the quantification of nMal d 1. Blots of three apple extracts with low, medium, and high Mal d 1 content as measured in ELISA, were incubated with pAb, mAb, BIP 1 (anti Bet v 1 mouse pAb kindly provided by K. Hoffmann-Sommergruber) and apple allergic patient sera (Mal d 1). Patient sera react mainly with a protein band at about 17 kDa. Rabbit pAb anti Mal d 1 reacts also with degradation products of Mal d 1.

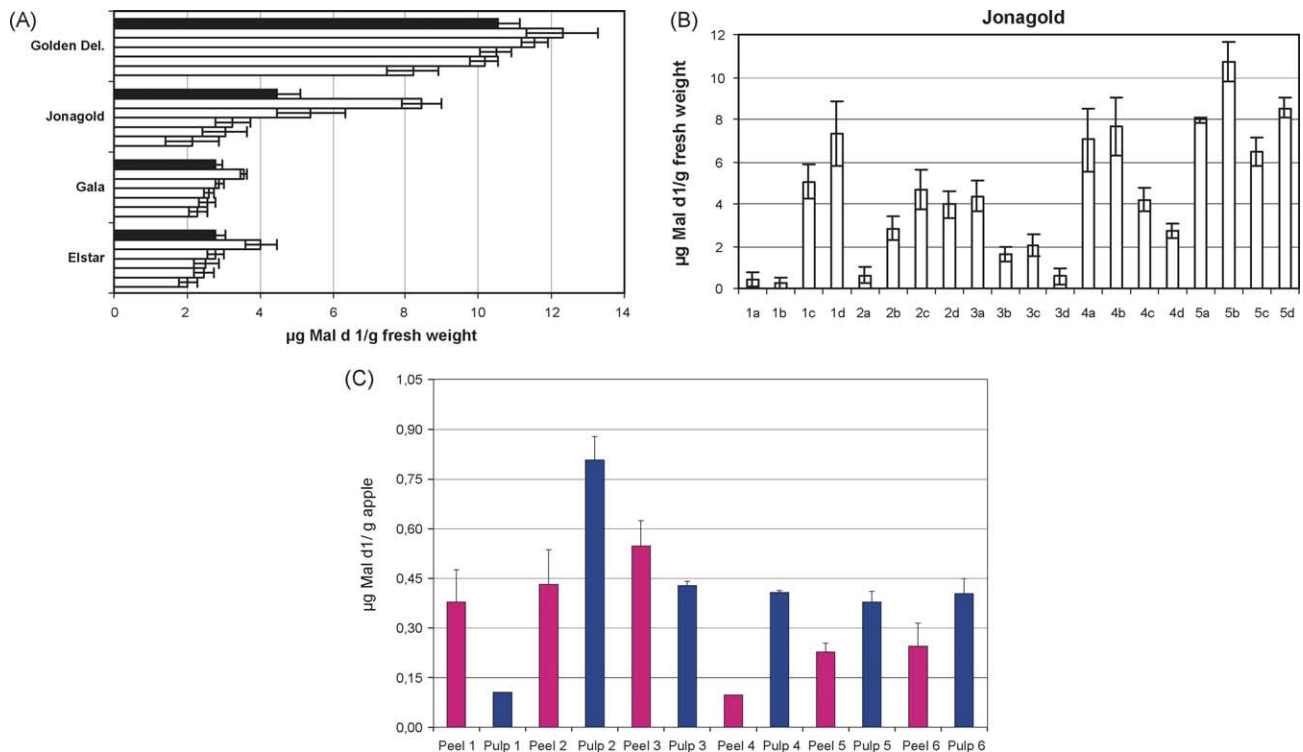


Fig. 5. Variability of Mal d 1 protein in different apple cultivars. (A) White bars represent individual apple (mean ± S.D. of four samples per apple), black bar represent mean of five apples. (B) Numbers indicate individual apple, letters indicate individual sample drawn per apple. Values represent mean ± S.D. of four independent measurements (freshly thawed aliquot was used for each testing). (C) Mal d 1 content of six Braeburn apples in peel and pulp. The apples were obtained from well-defined source, and indicated cultivar independent variability in distribution of allergen concentration in peel and pulp.

separated groups. Two cultivars seem to be definitely outliers (Stayman Striato, Mela Campanina). Genotypes cultured either conventionally or under organic farming conditions cluster in the same groups, e.g. G 185 and G198 in the low group and Fuji in the high group. Data obtained on sugar content, firmness, pH and malic acid did not correlate with the Mal d 1 expression level of cultivars (Marzban et al, in prep.).

4. Discussion

The importance of Mal d 1 for real food allergy in Northern and Mal d 3 in Central European countries has been demonstrated [3,18,21]. Mal d 1 is the major apple allergen in patients from Austria, The Netherlands and Northern Italy. More than 85% of patients from these countries are already sensitised to Mal d 1. The importance of Mal d 3 in central European countries has not yet been demonstrated. The allergen seems to be more relevant in Southern Europe (Italy, Spain) according to the data available [20,25].

This study shows a heterogenous expression and localisation of allergen transcripts and protein in apple fruits. All cultivars analysed so far express the major apple allergen proteins, Mal d 1 and Mal d 3. Therefore, we assume that there is no naturally occurring cultivar without

the respective allergens. Of course, the expression levels in different cultivars are significantly different, and therefore essential for patient reaction and their nutritive behaviour.

The prevalence of certain Mal d 1 transcripts in fruits and vegetative tissues of apple has been reported previously [16,26]. This study demonstrates that the occurrence of Mal d 1 isoforms is cultivar independent, and that differences between Mal d 1 transcripts are observed in different apple cultivars. Nevertheless, the expression pattern of major Mal d 1 isoforms seems to be similar. Investigations of the allergenicity of recombinant Mal d 1.01 (Mal d 1a) and Mal d 1.02 (Mal d 1b) revealed a higher IgE binding capacity for Mal d 1.01 [20]. Currently no data exist about the allergenic potential of Mal d 1.03 and hence, besides the fact that it is the least expressed isoform in all apple tissues, including fruits, the clinical relevance remains unknown.

ITP allows a clear mapping of allergens within apple tissues in agreement with Northern blot results. The peel-specific expression of Mal d 3 underlines its most likely involvement of nsLTPs in epicuticular wax or cuticle biosynthesis confirmed by its molecular structure [27] and in plant defense mechanisms, e.g. antimicrobial activities [25,20]. Recently little variations were detected in the Mal d 3 genes of 10 different apple cultivars [23]. The highly variable content of Mal d 1 observed in fruits has implications on allergen measurement and on the extract preparation for diagnostic purposes as well. Results from SPTs with

Table 1
Determination of Mal d 1 of 39 different apple cultivars

Cultivar	μg Mal d 1/g apple	S.D.	Soluble protein ($\mu\text{g/g}$)	% Mal d 1/total soluble protein
Low group				
Orim (43)	0.84	0.14	2083.5	0.04
G 362 (32)	1.32	0.57	1939.7	0.07
G185 (27)	1.40	0.39	1158.4	0.12
G185 Bio. (28)	1.43	0.38	1603.6	0.09
FG 240 (16)	1.57	0.23	1712.9	0.09
G 362 (31)	1.73	0.52	2033.0	0.08
SA 15/4 Saturn (50)	2.29	0.14	1751.0	0.13
Red Chief (47)	2.78	1.06	1777.8	0.16
G198 Bio. (30)	3.12	0.68	1876.7	0.17
8 SD 69 23 (2)	3.20	0.80	1524.9	0.21
Jonagold (38)	3.33	0.86	1742.4	0.19
G198 (29)	3.47	0.26	2037.5	0.17
Shizuka (51)	3.79	0.16	2134.5	0.18
BI 028 (6)	4.31	0.36	2015.9	0.21
FG 93 (19)	4.68	0.42	2401.2	0.19
Rubens Bio. (48)	4.91	0.73	1976.9	0.25
Medium group				
IH 64 (37)	5.23	0.83	1242.1	0.42
11/22 CH (1)	5.40	1.05	2005.1	0.27
FG 88 (18)	5.71	0.26	2487.4	0.23
DL 13 Bio. (10)	6.01	1.30	2022.3	0.30
DL 33 Bio. (12)	6.57	0.95	1480.3	0.44
Sundowner (53)	6.63	0.36	1954.9	0.34
Rubens (49)	6.72	0.61	1749.3	0.38
FG96 (21)	6.78	0.63	2001.0	0.34
FG96 Bio (22)	6.96	0.86	1744.4	0.40
Cameo (9)	7.89	0.41	1294.7	0.61
Braeburn (7)	8.08	0.73	1541.9	0.52
FG 260 (17)	8.32	1.12	2055.2	0.40
FG 93 Bio. (20)	8.51	1.19	2147.6	0.40
Braeburn Bio. (8)	8.81	0.47	1553.5	0.57
Pilot (45)	8.90	0.29	1690.5	0.53
High group				
Arlet (4)	9.15	1.38	1961.4	0.47
FG 114 (15)	9.60	0.96	2011.7	0.48
DI 24 Bio. (11)	9.80	0.59	1890.3	0.52
Enterprise (14)	10.74	0.41	1670.3	0.64
May Gold (41)	10.78	1.53	2308.8	0.47
Fuji (23)	11.50	0.40	4692.4	0.24
Fuji (N. fu 12) (24)	11.50	0.28	1827.0	0.63
Granny Smith (36)	12.14	3.58	1200.3	1.01
Primiera (46)	12.14	4.20	1691.9	0.72
Golden (34)	12.18	0.19	1657.5	0.73
LL 200 (40)	12.62	1.68	2141.3	0.59
Goldrush (35)	12.76	0.67	1273.2	1.00
Fuji Bio. (25)	12.84	2.17	2172.3	0.59
DL35 Bio. (13)	13.92	1.06	2189.7	0.64
Ambrosia (03)	13.99	0.90	1525.2	0.92
Pink Lady (44)	14.11	2.44	1783.6	0.79
LH 15 (39)	14.79	2.03	1767.1	0.84
Stayman Striato (52)	31.51	4.44	1076.0	2.93
Mela Campanina (42)	33.17	3.04	1550.8	2.14

Five apple per cultivar were homogenized, and extracted. Measurements were repeated four times. Standard deviation values (S.D.) demonstrate the variability of measured Mal d 1 contents. Apple cultivars organically grown are listed as (Cultivar name + Bio). Cultivars are ranked according to their Mal d 1 content and classified in low, medium and high groups.

birch-pollen sensitive patients revealed a higher allergenicity of extracts prepared from peel only in contrast to extracts prepared from the whole fruit of GS. A higher amount of Mal d 1 in peel and a better quality of peel extracts due to less contaminating polyphenolic compounds in Granny Smith was reported [29]. The fact that Mal d 1 is differently expressed in apple peel and pulp requires new strategies in sampling, and should be reflected in extract preparations or in performing SPTs using fresh apple tissue. Consumption of sufficiently heat-treated apples (at least 15 min at 100 °C) is recommendable to patients characterised to be solely reacting to Mal d 1. This solution is not applicable for Mal d 3 allergic patients, since the protein persists denaturation for 130 min at 180 °C [9]. For these patients apple peeling is a more effective strategy to escape an allergic response.

Analysis by ELISA allowed the analysis of 39 apple cultivars by their Mal d 1 content. Data obtained by a patient independent assay offer a first insight into allergen levels of different apple cultivars—ranging from 0.84 to 33.17 $\mu\text{g/g}$ fresh weight. Distinguishing three groups of high, medium and low, we find cultivars known as highly allergenic from clinical practice like Granny Smith or Golden Delicious in the high ranking group [24,30]. Given the fact that up to 18 isoforms of Mal d 1 were described [28] and considering that some of them exhibit different expression patterns in different tissues, e.g. fruit versus leaf [31], it is obvious that further investigations involving isolated isoforms are necessary, to finally unravel the biological function of these molecules. The influence of periodical, regional and cultivation-related factors on the level of allergen could not be completely elucidated in this investigation, but requires further studies with higher sample numbers. The value of a patient independent assay becomes even more evident, if seasonal fluctuation of patients allergic responses along the year are considered.

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