

Direct evidence for the presence of allergens in *Rosaceae* fruit tree pollen

G. Marzban · A. Mansfeld · A. Herndl ·
S. Jäger · M. E. Stoyanova · W. Hemmer ·
H. Katinger · M. Laimer

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Abstract The allergen content of insect-carried fruit tree pollen is poorly investigated. A recent report on orange allergy indicates a possible co-sensitisation to fruits by digestion and inhalation, suggesting an alternative mechanism for allergy development. Allergy to *Rosaceae* fruits like apple, pear, peach and cherry has been described as cross-reactivity to the birch pollen allergen Bet v 1. The expression of two major apple allergens Mal d 1 and Mal d 3 and their homologues in *Rosaceae* pollen was investigated. Transcript expression of five Mal d 1 isoforms and of Mal d 3 was examined by RT-PCR. A Mal d 1 specific ELISA was used to quantify the content of Mal d 1 homologues in *Pomoideae* pollen. Polyclonal antibodies against Mal d 1 and Mal d 3 were used for allergen detection in pollen extracts in Western blots. The cross-reactivity of pollen

allergens with patient sera was confirmed by IgE-Western blotting. Pollen counts were carried out by using Hirst-type volumetric samplers, revealing an intermittent *Rosaceae* fruit tree pollen load, without remarkable peaks across large parts of Europe. The expression of Mal d 1 was confirmed in pollen of all tested *Rosaceae* fruit tree species by ELISA, Western blot and RT-PCR. IgE-Western blots showed a strong cross-reactivity, particularly with Mal d 1 and Mal d 3 homologues in different *Rosaceae* pollen. Although the pollen load of *Rosaceae* is rather low as a rule, there is certified evidence for temporary peaks, indicating that allergen exposure for sensitized individuals is likely.

Keywords *Rosaceae* · ELISA · Western blot · SDS-PAGE · Fruit tree pollen · Bet v 1 · Mal d 1 · Mal d 3 · IgE reactivity · RT-PCR (Reverse transcription-polymerase chain reaction)

G. Marzban · A. Mansfeld · A. Herndl ·
M. E. Stoyanova · H. Katinger · M. Laimer (✉)
Plant Biotechnology Unit,
Department of Biotechnology, BOKU,
Vienna, Austria
e-mail: Margit.laimer@boku.ac.at

S. Jäger
HNO Clinic of Medical University of Vienna,
Vienna, Austria

W. Hemmer
Floridsdorfer Allergy Center, Vienna, Austria

1 Introduction

The observation of associative allergic reaction to pollen of birch tree and immediate type of food allergy to fruits confirms the general hypothesis of cross-sensitisation for most allergic patients (Caballero & Martin-Esteban, 1998; Dreborg, 1988). In the Northern hemisphere, up to 70% of birch pollen allergic individuals develop fruit

allergies to apple or other members of *Rosaceae* (Vieths, Scheurer, & Ballmer-Weber, 2002). Among pollen-related food allergies, the birch and grass pollen-associated ones are very important and have been studied extensively. It was claimed, that pollen-related food allergies in adults are more frequent than real food allergies (Eriksson, Formgren, & Svenonius, 1982; Etesanifar & Wütrich, 1998). Indeed, the most recent studies on the prevalence of adverse reactions to food in Germany confirmed the previous data and showed the highest rate of affected patients at the age of 40 years (Zuberbier et al., 2004).

Allergy to *Rosaceae* fruits and birch pollen is linked to cross-reactivity of IgE antibodies to Bet v 1, the major birch pollen allergen. In regions without birch vegetation, like Southern Europe and Japan, allergy to fruits is linked to grass, *Ambrosia* and *Artemisia* pollen or can be induced by pollen from other species related to birch like *Corylus*, *Alnus* and *Carpinus* (Fernandez-Rivas, van Ree, & Cuevas, 1997; Mari, Wallnert, & Ferreira, 2003; Wopfner et al., 2005; Yamagiwa et al., 2002).

The currently favoured assumption is, that apple sensitivity develops on the basis of primary sensitisation to birch pollen. Natural exposure to apple pollen has not yet been considered as inducing sensitisation. In fact, in contrast to the anemophilous birch pollen, apple pollen is insect-carried and thus unlikely to cause pollinosis (Vaughan, 1954). Since apple allergens have been detected in seeds, leaves, pulp and peels of apples (Marzban et al., 2005), the presence of cross-reacting allergens in pollen of apple trees seems plausible. First studies in fact revealed similar IgE cross-reactivity among the pollen proteins of birch and apple trees (Berrens, van Dijk, Houben, Hagemans, & Koers, 1990). Recently significant IgE-mediated allergy to papaya and orange tree pollen was demonstrated as a consequence of occupational fruit pollen exposure (Blanco et al., 1998; Iraneta et al., 2005).

In the current study we focused on the presence of different allergens in *Rosaceae* pollen, in order to get a first insight into expression patterns of major fruit allergens Mal d 1 and Mal d 3. Different methods were employed for specific detection of allergen proteins, including PCR,

Mal d 1 ELISA, IgG-Western blotting and patient IgE-Western blotting. These investigations demonstrate an alternative source of allergen and suggest a potential new route of sensitisation, particularly in the light of pollen data collected and compared across Europe.

2 Material and methods

2.1 Pollen material

Flowers were harvested from the orchard of the Institute of Pomology, BOKU (apple, pear, peach, apricot and cherry) and fence trees (birch, alder and hazel) in Vienna, during flowering time from January till May of 2005, according to the species. Pollen material was collected by manual separation. Samples were stored at -80°C prior to RNA and protein extraction.

2.2 Pollen concentration monitoring

Standardised volumetric pollen monitoring (Hirst, 1952) was performed by the European pollen information service. About 600 sites reported throughout a European network at weekly intervals. Due to the high morphologic similarity of different *Rosaceae* pollen, only the family classification was used and the patient-relevant index (annual total/number of days) calculated.

2.3 Protein extraction

About 100 mg pollen material was extracted in 1.5 ml distilled water at 4°C on ice for 60 min. The vials were vortexed every 10 min during extraction. Finally solutions were centrifuged for 10 min at 4°C and by 13.000 rpm (HERMLE Z230MR, Germany). Supernatants were stored at -20°C .

2.4 Total protein determination and Mal d 1 ELISA

Concentration of total protein was determined with the BCA protein assay kit (PIERCE, USA) according to manufacturer's instructions. All extracts were buffer-exchanged to PBS, using PD-10 columns (Amersham Biotech, USA)

before protein determination due to high contents of reducing agents expected in pollen tissue. ELISA was performed as described before (Marzban et al., 2005). Purified native Mal d 1 was used as standard. All determinations were run in duplicates and Mal d 1 amounts were converted to microgram Mal d 1 per gram fresh weight of pollen.

2.5 RNA isolation, reverse transcription, and PCR detection of pollen allergens

The Mal d 1 gene family in apple consists of 18 members (Gao et al. 2005). Expression of five isoforms was confirmed in ripe fruits of Royal Gala: Mal d 1.01 (A), Mal d 1.02 (B) (the most abundant allergen), Mal d 1.03E (E), Mal d 1.06A and Mal d 1.06B (Beuning et al., 2004). Mal d 1.06 is considered a genetic marker for molecular estimation of allergenicity in apple cultivars (Gao et al., 2005). Therefore the expression of fruit-expressed and marker-related Mal d 1 transcripts was analysed in apple pollen.

Total pollen RNA was isolated using RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. About 2.5 µg of total RNA was treated with RNase free DNase I (Roche) and subjected for reverse transcription using SuperScriptTM II RNase H-Reverse Transcriptase (Invitrogen). The obtained cDNA was diluted 1:1 and used as a template for PCR reactions.

PCR reactions were performed using the HotStarTaq DNA Polymerase (Qiagen) in a TRIO thermocycler (Biometra) according to the HotStarTaq DNA Polymerase handbook, and contained 2–4 µl diluted cDNA in a final volume of 25 µl. The sequences of primers used for amplification of Mal d 1 and Mal d 3 fragments are listed in Table 1. The degenerated primers MD1F-EcoRI and MD1R-BamHI (Pühringer, Zinöcker, Marzban, Katinger, & Laimer, 2003) recognize highly similar 5' and 3' regions of several Mal d 1 isoforms. Newly designed primers Mal d 1.06F/R recognize specifically the members of the gene cluster Mal d 1.06: A, B and C (Gao et al., 2005). Amplification conditions for these primers were: 95°C-15' hot start (95°C-1'/65°C-1'/72°C-1') 35 cycles, 72°C-5' final extension. PCR conditions for Mal d 3 (LTP) primers were: 95°C-15' hot start (94°C-1'/56°C-1'/72°C-1') 35 cycles, 72°C-5' final extension. About 7 µl of the PCR products were separated on a 2% agarose gel containing ethidium bromide and analysed.

2.6 IgG- and IgE-immunoblotting

Western blots were performed in a Novex-Gel apparatus using precasted 4–20% Tris/Glycin acrylamide gels (Invitrogen, Netherlands). Samples were denatured at 80°C for 10 min prior to analysis. After separation, proteins were transferred by electroblotting to a polyvinylidene difluoride

Table 1 Primers sequences and annealing temperatures (*T*) used for amplification of pollen allergens transcripts

Transcript	<i>T</i> (°C)	Primer sequence
Mal d 1 ^a	54	MD1F-EcoRI: NNNGAATTCATGGGTGTSTWCACATWYGAA MD1R-BamHI: NNNGGATCCTTAGTTGTAGGGRTCKGGTG
Mal d 1.01 ^a	56	Md1A-F: AAGCTGAAATCCTTGAAGGAA Md1A-R: GTGCTCTTCCTTGATTTCATG
Ma d 1.02 ^a	56	Md1B-F: ACACCTCTGAGATTCCACCAC Md1B-R:CAACTTGGTYTCGTAAGAGAC
Mal d 1.03 ^a	56	Md1E-F: ACCTCCGTCATCCCCCTG Md1E-R: TCTTCTCAATTGTCTCAGAGAT
Mal d 1.04 ^a	56	Md1D-F: CATCGAAGGCGATGGAGGT Md1D-R: CCTTAGCAYGGTAGTGGCTA
Mal d 1.06 ^b	65	Mal d 1.06-F: TGTCTCACATACGAAACCGAA Mal d 1.06-R: TGTGCTTCACATAGCTGTATTCACTC
Mal d 3 ^a	56	Mal d 3-F: ATGGCTWGCTCTGCARTGAYYAAG Mal d 3-R: TYACTTCACGGTGGCGCAGTTG

Primer names indicate the differences in the nomenclature of Mal d 1 isoforms according to Pühringer et al. (2003)^a and Gao et al. (2005)^b

(PVDF) membrane (Millipore, USA). First polyclonal antibodies against Mal d 1 (Marzban et al., 2005) or Mal d 3 (Herndl et al., submitted) were used, followed by anti-IgG rabbit antibodies labelled with alkaline phosphatase (Sigma, USA). Pre-stained Precision Plus Protein Standards (Biorad, USA) was applied as molecular weight marker.

IgE-immunoblotting was performed with sera of Austrian patients allergic to apple and birch using non-radioactive detection (A. Herndl et al., submitted). In brief, the separated pollen proteins were transferred to a nitrocellulose membrane (BIO-RAD, USA) and blocked in a PBS buffer (0.1% Tween/3% BSA), pH 7.4, for 2 h at 37°C. The membranes were incubated for at least 16 h at 4°C with 400 µl patient serum in 20 ml PBS buffer (0.1% Tween/1% BSA). After three times 5 min washing with PBS buffer (0.1% Tween), membranes were incubated with horse raddish peroxidase-labelled mouse anti human-IgE antibody (ZYMED, USA) (1:2000 diluted in PBS buffer 0.1% Tween/1% BSA) for 4 h at RT. After three times 5 min washing, the protein bands were detected with ECL Plus Western Blotting Detection Reagents (Amersham Bioscience, US) according to the manufacturer's protocol. Native Mal d 1 and Mal 3 were used as positive controls. Mal d 1 was purified using ion-exchange and size-exclusion chromatography. As ion-exchanger a Mono-Q column and as size-exclusion a Sephadex Column (Amersham, USA) were used. Purified Mal d 3 was obtained by a one step separation using a Mono-S column (A. Herndl et al., submitted).

3 Results

3.1 Tree pollen burden in European sites

During a period of three decades, pollen loads have been reported and analysed (Fig. 1). Analyses showed that the *Rosaceae* pollen concentrations reach noteworthy amounts and there exists a continuous level of *Rosaceae* pollen content across Europe, without drastic maximum peaks compared for example to birch pollen. Using *Rosaceae* pollen as common term, one should

keep in mind the geographic distribution pattern of the different botanical species (Tutin et al., 1978). In Northern Europe *Sorbus* sp. will prevail, while in Central Europe fruit tree species like *Malus*, *Pyrus* and *Prunus* and in Southern Europe *Prunus* ssp. will prevail in the sample.

3.2 Determination of total soluble protein and Mal d 1 content of apple pollen

Due to the intensive colour and high content of reducing substances, pollen extracts were buffer exchanged prior to protein determination: the ratio of Mal d 1 content to total pollen protein was determined by quantitative ELISA in apple and pear pollen (Fig. 2), using the monoclonal anti-Mal d 1 antibody 4C3H10, which apparently recognizes a distinct epitope conserved among the *Pomoideae*. The content of Mal d 1 in extracts of fruit pollen (245–775 µg/g fresh weight) is considerably higher than of fruit pulp (from 0.8 to 33 µg/g fresh weight) (Marzban et al., 2005). This may be explained by the high protein content of fruit pollen, which reaches concentrations up to 297 mg/g fresh weight (30%), while in apple pulp protein ratios from 0.3 to 0.5% of fresh weight are observed (Fig. 2).

3.3 Expression of Mal d 1 isoforms and Mal d 3 in apple pollen

RT-PCR demonstrated the presence of the Mal d 1 and Mal d 3 transcripts in pollen material of two apple cultivars 'Lavantaler' and 'Rheinischer Winterrambour' (Fig. 3A, G). PCR data indicated transcription of at least five different Mal d 1 isoforms and of Mal d 3 in apple pollen (Fig. 3B–F) and confirmed the results obtained at the protein level.

3.4 Immuno-detection of fruit pollen allergens using IgG and IgE antibodies

Pollen extracts were analysed by SDS-PAGE and Western blot. Using polyclonal rabbit antibodies against Mal d 1, we could detect for the first time Mal d 1 and homologue proteins in all pollen extracts (Fig. 4a). This means that Bet v 1 homologue proteins are expressed also in fruit tree

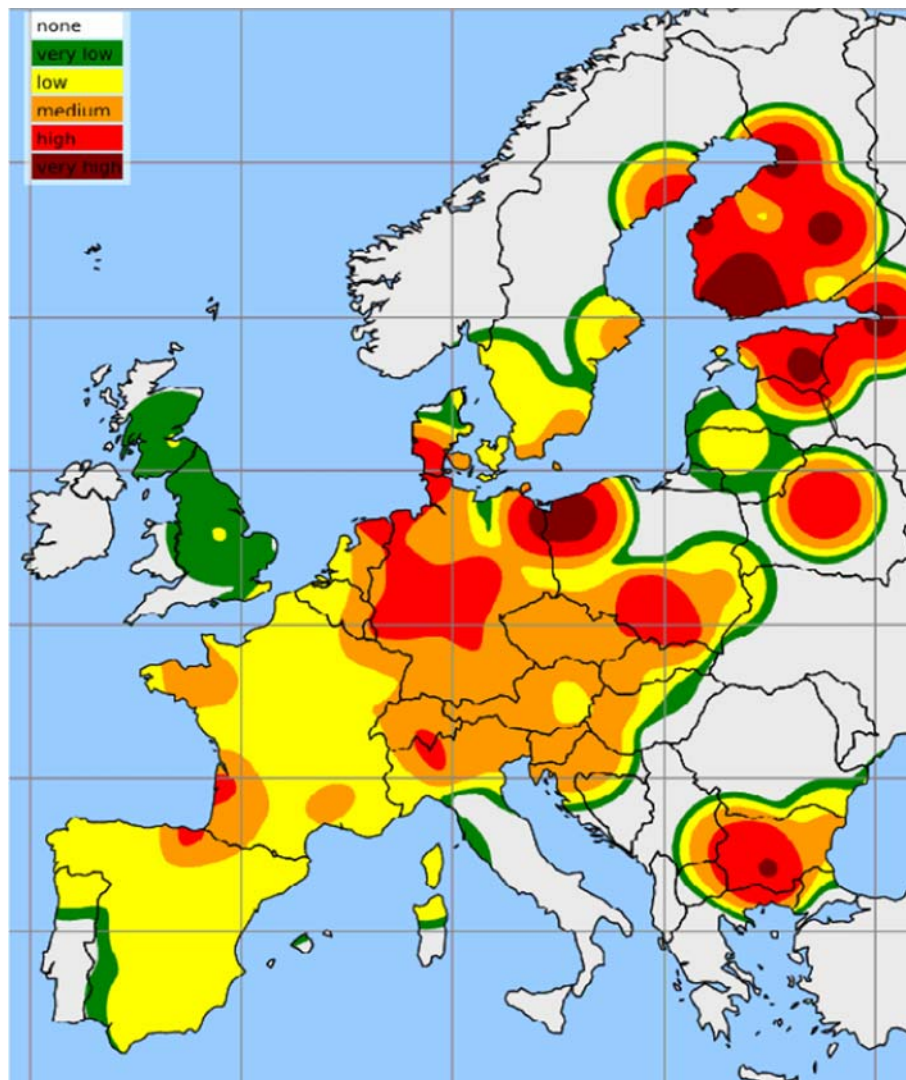


Fig. 1 *Rosaceae*-pollen index (annual total pollen counts divided by number of days with incidence)—none: 0–3; very low: 3; low: 13; medium: 29; high: 51; very high: 80. Data collected over large areas of Europe over a period of up to 29 years indicate considerable differences in the

exposure to the relevant pollen according to the geographic distribution of *Rosaceae* species. The high incidence in Northern Europe might be due to *Sorbus* sp., while in Central and Southern Europe mainly *Malus* and *Prunus* sp. are relevant

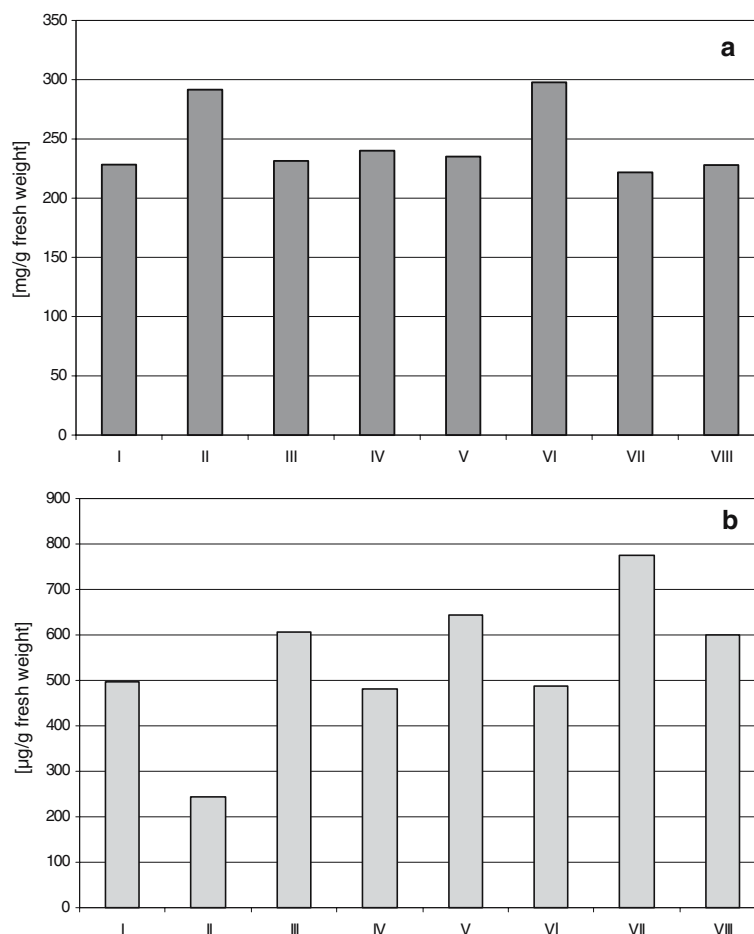
pollen, and not exclusively in pollen from birch and other *Fagales*. Likewise, Mal d 3 and homologous LTPs (Fig. 4b) were found in all pollen extracts of pome and stone fruits, while no signal was detected in the birch pollen extract.

Patient sera with well-documented clinical history in relation to birch pollen and apple fruit showed a positive reaction to Bet v 1 (Fig. 5). The patient sera with a defined history of severe systemic reactions after consumption of pome and

stone fruits, reacted with Mal d 1 and Mal d 3 homologues in different fruit pollen. The sera of three patients representing a typical Bet v 1 sensitisation pattern, recognized Mal d 1 homologues in pollen of apple, pear, apricot, peach, cherry and also with purified Mal d 1 as positive control. On the other hand, sera of two LTP-sensitized patients reacted with Mal d 1 homologues in apricot, peach and cherry (stone fruits), and with Mal d 3 homologues in apple, pear and peach.

Fig. 2 The content of total soluble protein (a) and Mal d 1 homologue allergens (b) in different apple and pear pollen.

I: *Malus domestica* cultivar Lavantaler;
 II: *M. domestica* cultivar Virginia Crab;
 III: *M. domestica* cultivar Golden Delicious;
 IV: *M. domestica* cultivar Gravensteiner;
 V: *M. pumila* (wild apple); VI: *M. domestica* cultivar Rheinischer Winterrambour;
 VII: *Pyrus communis* cultivar Delbard 51;
 VIII: *P. communis* cultivar Delbard 333



4 Discussion

The presence of allergens in *Rosaceae* pollen was so far not well documented, with the exception of one report on a Bet v 1 homologous protein in apple tree pollen (Berrens et al., 1990). Pollen from *Fagales* has been shown to be of high clinical impact due to airborne pollination. In contrast, it was assumed, that inhalation of insect-borne fruit tree pollen is only limited to people having a very close contact to fruit trees in the context of occupational exposure (Iraneta et al., 2005). Monitoring of *Rosaceae* pollen across Europe during the past 30 years, showed a moderate, but constant exposure. However, their concentration is low, when compared with birch pollen data.

The current study confirmed by three independent methods (ELISA, Western blot and RT-PCR), the expression of Mal d 1, Mal d 3

and their homologues in *Rosaceae* pollen. IgE-Western blots revealed a more complex pattern. Apple/birch allergic patient sera reacted with proteins in a range of 17–70 kDa. The fact, that also Mal d 3 homologues are found in *Rosaceae* pollen deserves special attention. Exposure to this protein source might be relevant for Northern and Central European countries, where allergy to fruit LTP is rare, while it might be less relevant in Southern Europe, where allergy to *Rosaceae* fruits is mainly caused by consumption of fruit LTPs. In fact the phenomenon, that allergy development of patients from different countries to a given fruit is driven by different allergens, has already been described in the case of cherry (Scheuer et al., 2001). According to previous reports, IgE-binding activities in higher molecular weight ranges (>30 kDa) are due to cross-reactive carbohydrate determinants (CCDs)

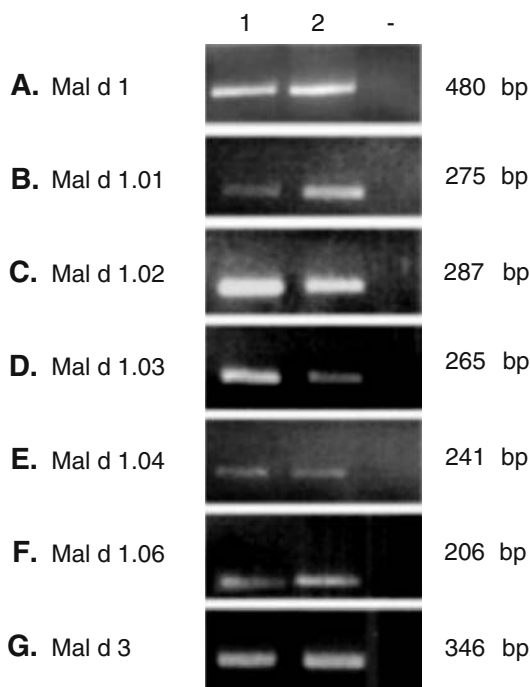


Fig. 3 Detection of allergen transcripts: Mal d 1 (a–f) and Mal d 3 (g) in apple pollen. Amplified transcripts are representative for all described subfamilies of Mal d 1 expressed in apple pollen: Mal d 1.01 and Mal d 1.02—subfamily I; Mal d 1.03—subfamily IV; Mal d 1.04—subfamily II; Mal d 1.06—subfamily III. PCR products amplified from cDNA were separated on a 2% agarose gel. The length of the PCR products is defined in base pairs (bp). Panels: (a) total Mal d 1 transcripts; (b) Mal d 1.01; (c) Mal d 1.02; (d) Mal d 1.03; (e) Mal d 1.04; (f) Mal d 1.06; (g) Mal d 3. Lane 1: pollen cDNA from cultivar Lavantaler; lane 2: pollen cDNA from cultivar Rheinischer Winterrambour; lane -: negative control (Aqua dest)

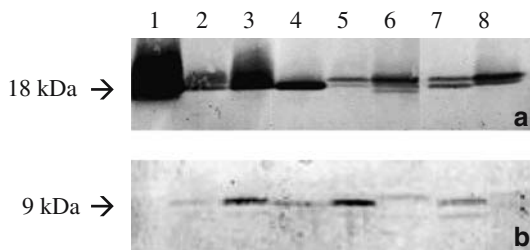


Fig. 4 Detection of the Mal d 1 (a) and Mal d 3 (b) in fruit pollen extracts by Western blot using polyclonal antibodies—1: *Betula* sp. (birch); 2: *Malus domestica* (cultivar Rheinischer Winterrambour); 3: *M. domestica* (cultivar Lavantaler); 4: *Pyrus communis* (pear); 5: *Prunus armeniaca* (apricot); 6: *P. persica* (peach); 7: *P. cerasifera* (sour cherry); 8: *P. avium* (cherry)

(Fötisch & Vieths, 2001; Petersen, Vieths, Aulepp, Schlaak, & Becker, 1996; Vieths et al., 2002), whose allergenic potential are still under study.

Data obtained indicate the presence of Mal d 1, Mal d 3 and their homologues in *Rosaceae* pollen. Mal d 1 was previously shown to be expressed in young and ripe apple fruits, old leaves and young leaves under stress conditions (Atkinson, Perry, Matsui, Ross, & MacRae, 1996; Beuning et al., 2004; Pühringer et al., 2000). Due to the limited size of samples and the low amount of total RNA extracted, we identified allergen transcripts using RT-PCR. The obtained results demonstrate that, as in the apple fruit, at least five members of Mal d 1 gene family are expressed in pollen, including the most abundant fruit-allergen Mal d 1.02 and Mal d 1.04, which was not found in the fruit. Since the expression of this protein is regulated by pathogen and stress, the presence of Mal d 3, a non-specific lipid transfer protein in apple pollen was astonishing. Further, the expression of nsLTPs could be demonstrated also in pear, peach and cherry pollen. nsLTPs have been identified as major allergens in apple and peach in patients not allergic to birch pollen (Pastorello et al., 1999; Sanchez-Monge, Lombardero, Garcia-Selles, Barber, & Salcedo, 1999) and cross-reactivity between nsLTPs in *Rosaceae* fruits, nuts, chestnut and maize has been observed (Asero et al., 2000). LTPs have also been identified in pollen of some important hayfever plants, such as mugwort (Art v 3), pellitory (Par j 2) and sycamore (Pla a 3). However, no or only limited cross-reactivity between these proteins and Mal d 3 and Pru p 3 has been found, leading to the view, that food LTPs primarily act as “true” food allergens (Diaz-Perales et al., 2000; Pastorello et al. 2002). The successful identification of Mal d 3 homologues in *Rosaceae* pollen in this study, along with the finding of a constant background fruit tree pollen load, might provide a new explanation for the origin of allergy to fruit LTPs.

Data presented in this study provide a new aspect of the allergen profile in *Rosaceae*, particularly for apple trees, which are the worldwide most produced and consumed fruits. A recent work on the phylogenetic relationship suggested that the

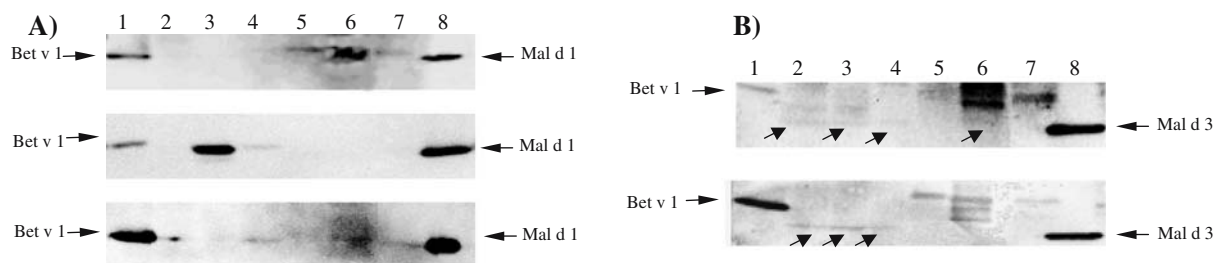


Fig. 5 Detection of Mal d 1 (**a**) and Mal d 3 homologues (**b**) in *Rosaceae* pollen was performed using IgE-immunoblot with sera of five birch/apple allergic patients. As positive controls purified native Mal d 1 and Mal d 3 (17.5 and 9 kDa) were applied to the gels. 1: *Betula* sp. (birch); 2: *Malus domestica* (Rheinischer Winterrambour); 3: *M. pumila* (wild apple); 4: *Pyrus communis* (pear); 5: *Prunus*

armeniaca (apricot); 6: *P. persica* (peach); 7: *P. cerasifera* (sour cherry); 8: positive controls. IgE-reactivity pattern to Mal d 1 homologues of three patients indicate individual differences. However, the IgE reactivity of two LTP-sensitized patients clearly appears in apple and pear pollen (the positive LTP bands are highlighted by arrows, →)

cross-allergenicity can reflect taxonomy in a majority of cases (Weber, 2003). Our results support, that most related fruit trees, e.g. pome fruits and stone fruits, express similar antigens with allergenic potential in their pollen. It is still to be clarified, if a baseline concentration of *Rosaceae* pollen in the atmosphere can be identified as a source for cross-sensitisation. Future studies are needed to clarify the cross-reactivity of pollen LTPs and their clinical significance for individuals with prolonged contact to higher concentrations of fruit pollen through their profession (farmers, orchard growers, greenhouse workers).

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