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ORIGINAL CONTRIBUTION

D.Y. Son
S. Scheurer
A. Hoffmann
D. Haustein
S. Vieths

Pollen-related food allergy: cloning and immunological analysis of isoforms and mutants of Mal d 1, the major apple allergen, and Bet v 1, the major birch pollen allergen

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D.Y. Son · S. Scheurer · A. Hoffmann
D. Haustein · Dr. S. Vieths (✉)
Paul-Ehrlich-Institut
Department of Allergology
Paul-Ehrlich-Str. 51-59
D-63225 Langen
Germany

Summary *Background:* Mal d 1, the major apple allergen, cross-reacts with IgE specific for the major birch pollen allergen, Bet v 1, and is responsible for birch pollen related food allergy to apple. Isoforms of Bet v 1 showing minor sequence variations display different binding capacity for specific IgE antibodies from allergic patients. Moreover, strain-dependent variation of allergenicity has been reported for apples.
Objective: To investigate the occurrence of strain-dependent isoforms of Mal d 1 which may differ in their allergenic potential, to obtain data on structures essential for binding of Mal d 1 to the antibody, and to gain insights into the structures responsible for its IgE cross-reactivity to Bet v 1.
Methods: The cDNA of Mal d 1 from various apple strains was amplified by a PCR strategy based on conserved regions of known Mal d 1-sequences, and sequenced. Two major isoforms of Mal d 1 were expressed as recombinant proteins and purified, as were different variants of the major birch pollen allergen, Bet v 1. Together with already existing recombinant birch pollen and apple allergens, these were subjected to allergenicity testing by IgE-immunoblotting, enzyme allergo sorbent test and dose related mediator release. ”Hot-spots” for IgE-reactivity were identified by site-directed mutagenesis.
Results: Twelve Mal d 1-clones were sequenced from 7 apple varieties and compared to 3 known Mal d 1 sequences. The clones were clustered into two groups, each showing a high degree of sequence identity to one of the known sequences and specific differences to the third sequence. No strain-specific sequences were identified. In contrast, apple strains with reported differences in allergenicity showed different expression levels of the major allergen. Immunologic testing of recombinant allergens revealed high IgE binding capacity of 2 major isoforms, named GD26 and GS29, with a slightly higher IgE binding capacity of GD26. Moreover, the allergenicity was similar to another rMal d 1 reported in the literature, representing the isoform divergent from our clones. Mutational analysis of our Mal d 1 allergens identified serine in position 111 as essential for IgE binding. Allergenicity was almost depleted by changing this residue into a proline. Moreover, the corresponding serine residue, present in position 112 of Bet v 1, was in a similar manner crucial for the allergenicity of the birch pollen allergen.
Conclusion: We conclude that divergent allergenicity of apple strains mainly depends on different expression levels of the major allergen. Introduction of a proline residue in position 111 of Mal d 1

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and in position 112 of Bet v 1 led to a drastic reduction of allergenicity of both the pollen and the food allergen, obviously also removing the cross-reactive epitope. Mutants with reduced IgE-reactivity but maintained T-cell reactivity may represent new candidates for a safer specific immunotherapy with reduced side-effects.

Key words Apple allergen – epitopes – IgE – isoforms – mutants – strains

Introduction

Fresh apples belong to the most important fruits causing birch pollen related food allergies (BPRF). Two recent investigations revealed adverse reactions to apples in 24/51 (47 %) (1) and 63/83 (76 %) (2) of birch pollen sensitised patients, respectively. IgE against Bet v 1 from birch pollen cross-reacts with the major apple allergen Mal d 1 (2, 3). Comparative testing of purified Bet v 1 and Mal d 1 has shown that the cross-reactive antibodies bind more than 1 order of magnitude stronger to the pollen allergen (3). The molecular relationships of both allergens have been confirmed by partial amino acid sequencing (4, 5) and by complete sequencing at the nucleic acid level (6-8), both of which revealed a high degree of 55-68 % amino acid sequence identity.

Differences in the allergenic potency between apple strains have been reported which may, in part, be due to different expression levels of the major allergen (9). Moreover, these divergent allergenicities could be explained by the existence of high and low IgE-binding strain-specific isoforms of Mal d 1, showing minor sequence variations. In the case of the homologous birch pollen allergen, Bet v 1 more than 20 isoforms have been described, most of which show more than 90 % amino acid sequence identity (10-12). Some of these isoforms, for example Bet v 1d, bind weakly to allergen-specific IgE-antibodies from birch-pollen-allergic patients (10). The three known Mal d 1 sequences share between 89.3 % and 97.5 % sequence identity at the amino acid level (6-8). The allergenicity of only one of these isoforms has been studied in detail, and this molecule bound strongly to specific IgE from apple-allergic patients (6).

Moreover, the epitopes of Mal d 1 have not been studied in detail. Several „classical“ food allergens such as αs1-casein and ß-lactoglobulin from cows milk (13-15), ovalbumin (16), the codfish parvalbumin (17), a 16 kDa rice allergen (13) and the major allergen from mustard (18) resist heating and processing and have been shown to contain sequential epitopes. In contrast, the allergenicity of apple quickly disappears after heating of the fruit. In addition, commercial apple extracts often lack any allergenic activity (3, 19). This rapid decrease of activity is most probably due to interactions between oxidised plant phenols and the apple allergen (20). Proteolytic fragments of Mal d 1 as well as synthetic peptides derived from the Mal d 1 sequence show little IgE binding activity, indicating the absence of sequence epitopes (own, unpublished data). This corresponds to evidence for the presence of conformational epitopes on the cross-reacting pollen allergen Bet v 1 (21), and the Bet v 1-related food allergen Pru av 1 from cherry.

The present study was undertaken to gain further insights in the molecular basis of birch pollen related allergy to apple, in particular the distribution and allergenicity of different isoforms. The cDNA encoding for Mal d 1 was amplified from total RNA of seven apple strains by polymerase chain reaction (PCR) using primers based on identical regions of the three previously known Mal d 1 cDNA sequences. In parallel, the presence of Mal d 1 in protein extracts from the same apples was investigated. In order to compare the allergenicity of Mal d 1 isoforms, two representative Mal d 1 sequences were expressed as bacterial recombinant proteins and subjected to extended serological allergenicity testing using a previously known, highly allergenic Mal d 1 (6) as reference. For further comparison, variants of Bet v 1 were also cloned and used in allergen assays. To study conformational epitopes, point mutations were introduced into Mal d 1 and Bet v 1 clones. Sequence positions and introduced residues were selected on the basis of known low IgE binding Bet v 1 isoforms and further data obtained in this study. Our results suggest that two major variants of Mal d 1 are widely distributed among apple strains and that an allergenic epitope responsible for cross-reactivity to birch pollen can be removed by a single point mutation.

Methods

Patients' sera

All sera were collected from birch pollen-allergic patients who reported oral allergy-like symptoms after the ingestion of fresh apples at the Hospital Borkum Riff, Department of Dermatology and Allergology (Borkum, Germany, Dr H. Aulepp). Diagnosis was based on a positive skin prick test to both birch pollen extract and apple extract and serological data (see below). Patients with positive test results in these assays, a clear anamnesis of spring pollinosis and oropharyngeal symptoms after ingestion of apples, were classified as being allergic to both birch pollen and fruit. Those who were included in the study had an enzyme allergo sorbent test (EAST) class of at least 2 to both purified Bet v 1 and Mal d 1 which indicated the presence of significant amounts of serum-IgE specific for these allergens. Serum from a volunteer with no history of pollen and food allergy was used as control. All sera were stored at -20°C until used.

Monoclonal antibody

The monoclonal antibody (mab) 1D6 was obtained after immunisation of BALB/c mice with purified Mal d 1 (5) and belonged to the IgG1 subclass. The antibody was kindly donated by Drs. B. Fahlbusch and W.-D. Müller, University of Jena, Department of Clinical Immunology.

Allergenic extracts and allergens

Mature fresh apples were purchased at the local market. The apple strains Golden Delicious, Granny Smith, Jonagold, Idared, Royal Gala, Jamba, and Gloster were selected for this study. Apple extracts were prepared by a low temperature „acetone powder" method as described (3, 4). Freeze-dried birch pollen extract was purchased from Allergopharma (Reinbek, Germany). Mal d 1 and Bet v 1 were isolated by preparative sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by electroelution as described (3) and stored freeze-dried at -20°C. Recombinant Bet v 1a (22), the highly allergenic main isoform of the major birch pollen allergen and one isoform of Mal d 1 (termed Mal d 1c in the following) (6) were purchased from BIOMAY, Linz, Austria. The Bet v 1 mutant Bet v 1a M139L was a gift of Prof. P. Rösch, University of Bayreuth, Germany. Additional Mal d 1 and Bet v 1 isoforms and mutants were cloned and produced as bacterial recombinant proteins as described below.

Determination of specific IgE

Bet v 1 and Mal d 1 were coupled to cyanogen bromide activated filter paper disks (Hycor, Kassel, Germany) at a protein concentration of 1.2 µg/ml. Allergen extracts were diluted to a final concentration of 12 µg/ml prior to coupling. Specific IgE was measured by a commercially available EAST according to the instructions of the manufacturer (Allergopharma, Reinbek, Germany). The results were expressed as EAST classes (corresponding to the classes of Phadezym™ RAST (Pharmacia, Uppsala, Sweden)) or as arbitrary units per milliliter (U/ml).

Electrophoresis

Allergen extracts were separated by SDS-PAGE (T = 13 %, C = 2.7 %); gel size 180 x 170 x 1 mm^3, according to the method of Laemmli (23). This system was also used for monitoring the purity of isolated natural and recombinant allergens. After separation, proteins were either subjected to Coomassie brillant blue or silver staining or used for immunoblotting as described below. For calculation of the Mal d 1 content in apple extracts, Coomassie-stained gels were recorded with a digital documentation system (Digi-Store-Duo, Göttingen, Germany). The Mal d 1 content was calculated by taking the band intensity of the total protein pattern as 100 % level using the program Gel-Script (BioSciTec, Marburg, Germany).

Western blotting, protein staining and immunologic detections

Proteins and allergen extracts were transferred to nitrocellulose- (NC) membranes (Schleicher und Schüll, Dassel, Germany) by semi-dry blotting (24) as described (25). Transfer was controlled by staining single NC-strips of 4 mm with 0.1 % (wt/vol) India ink (Pelikan, Hannover, Germany) in PBS containing 0.05 % (vol/vol) Tween 20 and 1 % (vol/vol) acetic acid, or with an Auro Dye staining kit (Auro Dye™ forte, Amersham, Buckinghamshire, UK). Immunodetection was performed with 0.05 ml of patient serum diluted to 1 ml as described (3). The mab 1D6 was applied at 1 ml and antibody binding was detected as described (3), with the exception that the colorimetric substrate was replaced with chemiluminescence (ECL) using an ECL Kit (Amersham, Buckinghamshire, UK). All dilutions were made in 0.01 mol/l PBS containing 0.1 % bovine serum albumin (BSA) and 0.05 % (vol/vol) Tween 20. Blocking buffer was 0.3 % Tween 20 in PBS. For specific purposes, multiple transfers from one gel containing purified recombinant allergens were accomplished by thermoblotting: NC-membranes were placed on both sides of the gel and covered with wetted filter paper. Thereafter, the blot sandwich was incubated above the surface of a waterbath heated to 70°C, for 10 min. Up to three transfers were carried out from one gel. The incubation was continued for additional 2 min and 4 min for the second and third transfer, respectively.

Preparation of RNA and cDNA synthesis

Total RNA was isolated from birch pollen (Allergon, Engelholm, Sweden) and from the same apples that were used for allergen extract preparation by means of the RNeasy Plant Total RNA kit (Quiagen, Hilden, Germany) according to the manufacturer's instructions. The amount of RNA was estimated from the OD_{260}. RNA was reversely transcribed by the First Strand cDNA Synthesis kit (Pharmacia, Freiburg, Germany) according to the protocol of the supplier. First strand cDNA synthesis was primed with oligo–dT_{18} (ARK, Darmstadt, Germany). Approximately 0.5 µg of total RNA were used for each reverse transcription (RT). Products of the reverse transcription were directly subjected to PCR.

Mal d 1-PCR and rapid amplification of cDNA ends (5'RACE)

For 5'RACE, the RT reaction was primed with a gene specifc primer AM4: 5'-ATT AAT TTA GTT GTA-3'. AM4 represents the 3'-end of the coding region including six conserved nucleotides of the untranslated region. The

AM4 sequence is identical in the three published Mal d 1 sequences (6-8). To identify a consensus sequence at the 5'-end and to clone the unknown 5'-end of the cDNA, 5'RACE was performed with the GIBCO BRL 5'RACE system (GIBCO BRL, Eggenstein, Germany). A homopolymeric dC-tail was ligated to the 3'-end of the purified RT product by a terminal desoxynucleotidyl transferase reaction. The dC-tailed cDNA was amplified by PCR using a nested second antisense gene specific primer MMU, again representing a consensus element: 5'-GCA ATC TTB GGG ATG AGG-3' with B = T, C or G, and the abridged anchor primer provided with the 5'RACE system. The PCR conditions were: Hot start (94°C, 2 min), denaturation 94°C, 1 min, annealing 55°C, 1 min, extension 72°C, 2 min. After 35 cycles the mixture was cooled to 4°C. If the agarose gel electrophoresis revealed an indistinct band after the first PCR, the product was excised from the gel, purified by the QUIAEX II gel extraction kit (Quiagen, Hilden, Germany), and subjected to a second PCR using the abridged universal amplification primer provided with the 5'RACE system and again MMU as antisense primer.

A cDNA fragment of Mal d 1 that contained the entire encoding region was amplified with the universal gene specific 5'-primer AM5: 5´-ATG GGT GTC TAC ACA TTT GAG-3´ (nucleotides 1-21 of the coding region). For the reverse direction an oligo-dT-deg was applied, thus enabling the confirmation of the AM4 sequence for all apple varieties. PCR was performed in a final volume of 50 µl using a TRIO thermocycler (Biometra, Göttingen, Germany). Five µl RT product were added to a mix containing 0.25 mM of each dNTP (Pharmacia, Freiburg, Germany), 40 pmol of each primer and 2.5 U Taq polymerase (5 U/µl, Perkin Elmer Applied Biosystems, Weiterstadt Germany) and 15 µl (10x) Taq buffer (100 mM Tris-HCl, 500 mM KCl, 15 mM $MgCl_2$, pH 8.5, Pharmacia). The PCR conditions were: denaturation 94°C, 1 min, annealing 50°C, 1 min, extension 72°C, 1 min. After 35 cycles the final extension was 5 min at 72°C. To exclude laboratory contamination of apple RNA or PCR products between samples from different strains total RNA was prepared from kiwi fruit and amplified in parallel with apple RNA using the terminal sense and antisense primers.

Cloning and sequencing of Mal d 1 cDNA

PCR products were subjected to agarose gel electrophoresis. Purified DNA fragments were ligated into the pGEM-T vector (Promega, Madison, WI, USA) and used for transformation of *E.coli* XL1-Blue (Stratagene, Heidelberg, Germany) by electroporation (2.5 kV, 25 µF, 200 Ω) using the E.coli gene pulser (Biorad, Munich, Germany). Positive clones were selected by blue/white screening and controlled by PCR screening using the vector-specific primer SP6: 5'-GAT TTA GGT GAC ACT ATA GA-3' and AM5 as gene specific 5'-primer. Plasmids were isolated from overnight cultures by means of the Plasmid Midi kit (Quiagen, Hilden, Germany) and subjected to sequence analysis using an ABI 373 „stretch" automated fluorescent sequencer (Applied Biosystems, Weiterstadt, Germany) using SP6 and T7-promotor: 5'-TAA TAC GAC TCA CTA TAG G-3', as sequencing primers.

Synthesis of the encoding regions of Bet v 1 and Mal d 1 cDNA

In accordance with the data obtained from nucleotide sequencing, the entire encoding region was amplified from apple total RNA using the 5'-primer AM5 and the 3'-primer AM4. These primer regions were conserved between all apple PCR products that were sequenced. For birch pollen, the RT reaction was primed from 0.3 µg of total birch pollen RNA using an oligo-dT primer. The encoding region was amplified using terminal primers based on the known nucleotide sequence of the highly allergenic isoform Bet v 1a (22): 5'-Bet A: 5'-ATG GGT GTT TTC AAT TAC-3', 3'-Bet B: 5'-TTA GTA GGC ATC GGA GTG-3'. PCR products of about 500 bp were ligated into pGEM-T and again used for transformation of *E.coli* XL1-Blue cells. Positive clones were identified by PCR screening with primers T7-promotor and AM4 for Mal d 1 and with T7-promotor and 3´-Bet B for Bet v 1. After plasmid purification, the samples were again confirmed by sequencing.

Site-directed mutagenesis of Bet v 1

Site-directed mutagenesis of Bet v 1 was performed using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene, Heidelberg, Germany). The oligodeoxynucleotide primers BET-P151L-Back: 5´-TTA GTT GTA GGC ATA GGA GTG TGC CAA GAG GTA GCT CTC AAC GGC-3´ and BET-P151L-For: 5´-GCC GTT GAG AGC TAC CTC TTG GCA CAC TCC GAT GCC TAC AAC TAA-3´ were applied for amplification of Bet v 1 (S112P) using the plasmid Bet v 1 (S112P,L151P) as template. All oligodeoxynucleotide primers were HPLC purified and purchased from ARK, Darmstadt, Germany. PCR was performed at 95°C, 30 s followed by 15 cycles of 95°C, 30 s, 55°C, 60 s, 68°C, 12.4 min. Thereafter, the reaction mix was cooled to 4°C. The Bet v 1 (S112C) variant was amplified by a triple PCR strategy using the clone Bet v 1 (S112P,L151P) as template. The following primers were used: Bet-Nde-For: 5´-GGA ATT C*CA TAT G*GG TGT TTT CAA TTA CGA AAC TGA G-3´ and Bet-P112C-Back: 5´-CTT GTT GCT GAT CTT CAA GAT GCA TCC TCC ATC AGG GGA-3´; Bet-P112C-For: 5´-ACC CCT GAT GGA GGA TGC ATC TTG AAG ATC AGC AAC AAG-3´ and Bet-Xho-P151L-Back: 5´-CCG *CTC GAG* TTA GTT GTA GGC ATC GGA GTG TGC CAA GAG GTA-3´. Base pair substitu-

tions are underlined; the Xho I or Nde I cleavage site are printed in italics. An initial PCR (30 cycles) was performed for 1 min at 95°C, 1 min at 50°C, 1 min at 72°C, 1 min at 68°C and 5 min at 65°C as final extension. After a hot start, an additional assembly PCR of the two purified overlapping cDNAs was performed at 1 min at 94°, 1 min at 50°C, 1 min 72°C. After 8 cycles terminal primers were added and additional PCR (30 cycles) was performed at 1 min 94°C, 1 min 50°C, 1 min 72°C and 65°C as final extension.

Site-directed mutagenesis of Mal d 1

Single amino acid substitutions (S111P, S111C) were performed by site-directed mutagenesis of Mal d 1 by a triple PCR strategy. Genetic engineering of Mal d 1 was performed with the oligodeoxynucleotide primers Mal-Nde-For: 5´-GGA ATT C*CA TAT G*GG TGT CAT CAC ATT TGA GAA CGA G-3´, Mal-Xho-Back: 5´-CCG *CTC GAG* TTA GTT GTA TGC GTC GGG GTG GCC-3´, GD-C-For: 5´-TGT GGA AGT GGT TGC ACC ATC AAG AGC ATC AGT CAT-3´, GD-C-Back: 5´-ATG ACT GAT GCT CTT GAT GGT GCA ACC ACT TCC ACA-3´, GD-P-For: 5´-TGT GGA AGT GGT CCC ACC ATC AAG AGC ATC AGT CAT-3´, GD-P-Back: 5´-ATG ACT GAT GCT CTT GAT GGT GGG ACC ACT TCC ACA-3´, GS-C-For: 5´-TCT GGA AGT GGT TGC ATC ATC AAG AGT ATC AGC CAC-3´, GS-C-Back: 5´-GTG GCT GAT ACT CTT GAT GAT GCA ACC ACT TCC AGA-3´, GS-P-For: 5´-TCT GGA AGT GGT CCC ATC ATC AAG AGT ATC AGC CAC-3´, GS-P-Back: 5´-GTG GCT GAT ACT CTT GAT GAT GGG ACC ACT TCC AGA-3´ using Mal d 1 cDNA from GD and GS as template. Base pair substitutions are underlined, the Xho I and Nde I cleavage sites are printed in italics. PCR was performed as described for site-directed mutagenesis for generating Bet v 1(S112C). After Xho I and Nde 1 cleavage the purified cDNA was cloned into plasmid pET-15b (Novagen, Madison, WI, USA). Initial cloning was done in *E.coli* XL1-Blue (Stratagene, Heidelberg, Germany). Positive clones were selected by PCR screening with the oligodeoxynucleotide primers T7-promoter and Mal-Xho-Back. All oligodeoxynucleotide primers were HPLC purified and purchased from ARK, Darmstadt, Germany.

Expression of recombinant allergens and mutants

Using pGEM-T-plasmids as templates, 5'-Nde I and 3'-Xho I restriction sites were added to the encoding regions of the Mal d 1 and Bet v 1 cDNA by PCR using the primers Mal-Nde-For: 5'-GGA ATT C*CA TAT G*GG TGT CAT CAC ATT TTG AGA ACG AG-3', Mal-Xho-Back: 5'-CCG *CTC GAG* TTA GTT AGT TGT ATG CGT CGG GGT GGC C-3', Bet-Nde-For: 5'-GGA ATT C*CA TAT G*GG TGT TTT CAA TTA CGA AAC TGA G-3' and Bet-Xho-Back: CCG *CTC GAG* TTA GTT GTA GGC ATC GGA GTG TGC CAA GAG GTA-3' (restriction sites in italics). The products were digested with the respective restriction endonucleases and ligated into the expression vector pET-15b (Novagen, Madison, Wi, USA) which encodes for a 6-residue His-Tag fusion protein, amplified in XL1-Blue cells, purified and transformed into *E.coli* BL21(DE3) expression cells (Novagen). Positive clones were identified by PCR screening. Single colonies were grown at 37°C. Protein synthesis was induced by adding isopropyl-D-thio-galactopyranoside (IPTG) to LB-medium to a final concentration of 1 mM, and the incubation was continued for 6 h at 37°C. Cells were harvested by centrifugation, resuspended in lysis buffer (50 mM NaH_2PO_4, pH 8, 150 mM NaCl, 50 mM $MgCl_2$, 0.75 mg/ml lysozyme, 250 U benzonase, 5 mM EDTA, 1 % NP-40), and again centrifuged. Thereafter, the cell pellet was incubated in binding buffer: 20 mM Tris-HCl, pH 7.9, 500 mM NaCl; 5 mM imidazole) containing 6 M urea for 1 h at 4°C, and again centrifuged. The supernatant was incubated with Ni-NTA-Agarose (Quiagen, Hilden, Germany) at RT for 1 h. The Ni-NTA-agarose-bound fusion protein was washed three times with binding buffer containing 4 M urea, with binding buffer containing 2 M urea, and finally with pure binding buffer. To obtain non-fusion protein by enzymatic cleavage, 1.5 µl (0.6 U/µl) of biotinylated thrombin (Novagen) was diluted with 1 ml of binding buffer and soaked into a column containing bound fusion protein from 200 ml of bacterial culture. The sealed column was incubated overnight at ambient temperature. Non-fusion protein was eluted stepwise with 6 fractions of binding buffer containing 6 M urea. In the case of poor cleavage rates (<10% of total fusion protein), the digestion and elution procedure was repeated up to three times. Eluted fractions were controlled by SDS-PAGE, pooled, and sequentially dialysed against PBS containing 5 M, 3 M, 1 M and 0.5 M urea, respectively using a cut-off of 3.5 kDa and a period of 6 h for each dialysis step. Finally the allergens were dialysed overnight against pure PBS. The protein content of all preparations was estimated by a commercial dye binding assay (Pierce, Rockford, USA). The non-fusion allergens were stored freeze-dried at –20°C until used.

Rat basophil leukeamia- (RBL) cell mediator release assay

The RBL-cell mediator release assay has been developed as a murine model that mimics an essential event of the type I allergic reaction (26, 27). Briefly, specific IgE was raised against birch pollen in BALB/c mice by intraperitoneal injections with allergen extract. Sera from at least five mice were pooled and used for passive sensitisation of RBL-2H3 cells. Subsequently, the RBL-cells were incubated with serial allergen extract dilutions to induce a mediator release. The specific release was assayed by

measurement of ß-hexosaminidase activity in the supernatant. The total release was quantified by cell-lysis with Triton X-100. Controls were run with buffer without allergen to measure spontaneous release, and by stimulation of cells with antiserum of divergent specificity (e.g. anti-grass pollen IgE). Results were expressed in % of the total release after subtraction of the spontaneous release (26).

Similarity searches and sequence alignments

Similarity searches and alignments of deduced amino acid sequences were performed by non-redundant searches using the basic local alignment search tool (BLAST) server (28).

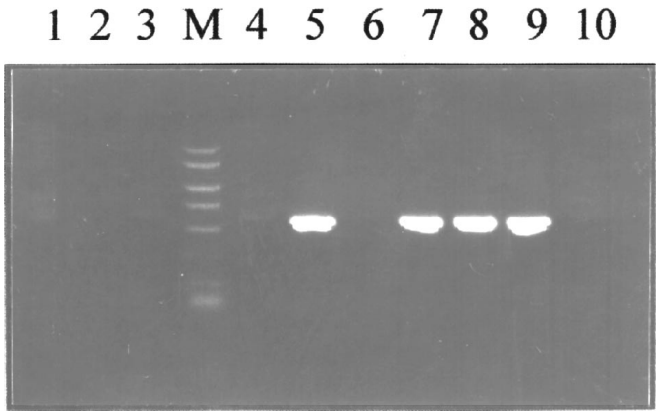

Fig. 1 PCR product of the Mal d 1 cDNA. *E.coli* XL1-Blue were transformed with pGEM-T plasmid containing the Mal d 1 cDNA as insert. Positive clones were identified by blue/white screening and screened by PCR using AM4 and T7-promotor as primers. Products were analysed by agarose gel electrophoresis. A characteristic 550 bp Mal d 1 insert was identified in the clones shown in lanes 5, 7, 8 and 9. M: PCR-Marker-DNA. Positive clones were further subjected to sequencing.

Results

Cloning and sequencing of Mal d 1 reveals the existence of two major isoforms

Total RNA was prepared from seven apple strains and subjected to reverse transcription using AM4, as antisense gene specific primer representing an identical partial sequence at the 3'-end of the coding region of the published Mal d 1cDNAs (6-8). Thereafter, the 5'-untranslated regions and the N-terminus of the coding sequence were amplified by 5'-RACE using the antisense gene specific primer MMU. According to the results of sequencing the 5'-gene specific primer AM5 was selected on the basis of a partial sequence shared by the 5'RACE products from all seven apple varieties. AM5 was also identical with two of the published Mal d 1 cDNAs (7, 8), but showed a single nucleotide exchange to the third published isoform (6).

Another Mal d 1 cDNA fragment covering the entire encoding region and the 3'-end of the the DNA was amplified by PCR with primer AM5 and oligo-dT. When analysed by agarose gel electrophoresis, the products from all apple strains revealed a prominent band of the expected size (approximately 750 bp consisting of 480 bp coding region and about 260 bp untranslated region). The purified products were cloned and sequenced as above. Since AM4 and AM5 represented the 3'-end and the 5'-end of the encoding region, respectively, these primers were used to amplify the entire coding region. Clones containing an insert of about 500 bp were subjected to PCR-screening and full-length sequencing. The result of a typical PCR screening analysis of clones containing the entire coding sequence from Gloster apples is shown in Fig. 1. Similar results were obtained from all seven apple varieties. To exclude laboratory contamination with apple RNA or amplified apple DNA, kiwi fruit RNA and apple RNA were tested simultaneously by PCR using the two terminal Mal d 1-gene specific primers. A distinct PCR product of about 500 bp was obtained for apple, whereas no PCR product was obtained with kiwi fruit RNA (result not shown).

Full-length coding sequences were obtained from 12 Mal d 1-clones derived from seven apple strains. Fig. 2 shows the aligned deduced amino acid sequences of all clones. For comparison the previously published Mal d 1-sequences are also shown. In the following, these three isoforms are named Mal d 1a (8) (GenBank Acc.: X83672), Mal d 1b (7) (GenBank Acc.: L42952) and Mal d 1c (6) (GenBank Acc.: Z48969) in order to reflect their importance as allergens. Six clones were almost identical with the Mal d 1a that has been sequenced from Granny Smith apples: clone JA10 from Jamba, clone GD26 from Golden Delicious, clone GD24 from Golden Delicious, clone GL6 from Gloster, clone GA18 from Gala and clone JG31 from Jonagold. Minor sequence deviations to Mal d 1a were found in position 134 of all clones (A instead of V). Moreover, clones GA18 and JG31 showed Y instead of F at position 9, clones JA10 and GA18 had D replaced with G at position 152, GA18 had an E/A exchange at position 137 and finally, clone JG31 presented a S/P exchange at position 16 of the deduced amino acid sequence. For these six clones, the sequence identity to Mal d 1a ranged between 97.5 % and 99.4 %. The number of deviating amino acids was one for clones GD26, GD24 and GL6, two for JA10, three for JG31, and four for

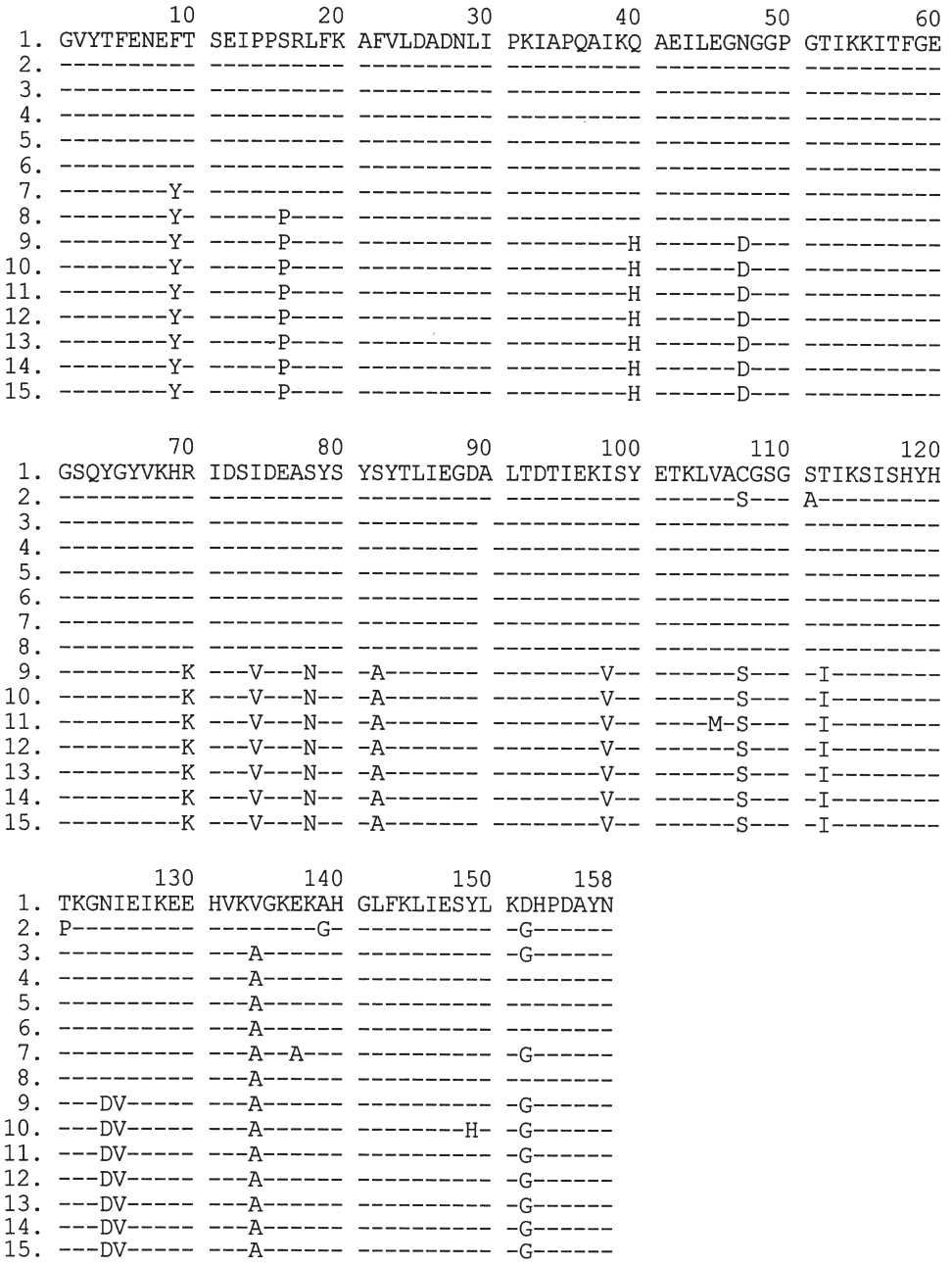

Fig. 2 Deduced amino acid sequences of Mal d 1 cloned from seven apple strains. Residues identical with Mal d 1a are marked by "-". (1) Mal d 1a from Granny Smith (8) (Acc.: X83672), (2) Mal d 1c from Golden Delicious (6) (Acc.: Z48969), (3) JA10 from Jamba, (4) GD26 from Golden Delicious, (5) GD24 from Golden Delicious, (6) GL6 from Gloster, (7) GA18 from Gala, (8) JG 31 from Jonagold, (9) Mal d 1b from Golden Delicious (7) (Acc.: L42952), (10) GD25 from Golden Delicious, (11) GL7 from Gloster, (12) GA17 from Gala, (13) GS29 from Granny Smith, (14) GS30 from Granny Smith, (15) IR29 from Idared.

GA18. On the contrary, clones GD25 from Golden Delicious, GL7 from Gloster, GA17 from Gala, GS29 from Granny Smith, GS30 from Granny Smith and IR29 from Idared corresponded closely to the Mal d 1b-sequence originally obtained from a Golden Delicious cDNA library. Mal d 1b contains 15 amino acid exchanges when compared to Mal d 1a. The six clones where identical with Mal d 1b except for GL7 containing an V/M exchange at position 105 and GD25 with Y changed to H at position 149. Mal d 1c is highly homologous to Mal d 1a, but shows five deviating amino acids at positions 107, 111, 121, 139 and 152. These key residues were not present in our Mal d 1a cluster of sequences, except for clones JA10 and GA18 which contained a G instead of D at position 152 which is also present in Mal d 1c. However, a G152 is also found in Mal d 1b and is therefore not characteristic for Mal d 1c. The amino acid sequence identities of our cloned Mal d 1 isoforms with Bet v 1 a ranged between 56.2 % and 56.9 %. In summary, our sequence data revealed that Mal d 1a and Mal d 1b are the dominating isoforms which occur widely distributed among apple varieties. The lack of characteristic residues of the Mal d 1c sequence may indicate that this isoform is of minor importance.

The cloned Bet v 1 isoforms show significant differences from the highly allergenic major isoform Bet v 1a

Three full-length sequences of the Bet v 1 encoding region, named B1, B2 and B3, were obtained by PCR with terminal primers 5'-Bet A and 3'-Bet B which were derived from the known nucleotide sequence of the highly allergenic isoform Bet v 1a (22). The deduced amino acid sequences are depicted in Fig. 3, lanes 3-5. For comparison the Bet v 1a sequence is shown in lane 1 and the sequence of Bet v 1 d which has been described as low IgE binding isoform is presented in lane 2. All three Bet v 1 clones showed minor sequence deviations when compared to the known Bet v 1 isoallergens. The degree of sequence identity with Bet v 1a and Bet v 1d ranged between 93.8 % and 98.8 % (Table 1). Clone B1 presented a high degree of 98.1 % sequence identity with Bet v 1d with only three differing amino acid positions. Interestingly, clone B1 contained the CV-motif in position 112/113 which has been described as characteristic for low IgE binding isoforms and mutants of Bet v 1 (29). Clone B2 was 98.8 % identical with Bet v 1a, containing only 2 diverging amino acid residues: P112 instead of S112 and P151 instead of L151. Clone B3 consisted of a mixed sequence of the known Bet v 1 isoforms, containing seven amino acid differences to Bet v 1a. One residue, L85, was different from all known Bet v 1 isoforms. Since position 112 appeared to be important for the IgE binding ability, clones B1 and B2 represented potentially low IgE binding isoforms and were selected for expression.

Construction of mutated allergens for epitope analysis

Site-directed mutagenesis was accomplished by PCR using mutated primers. The corresponding positions 111 of Mal d 1 and 112 of Bet v 1 were selected for point mutations. This decision was based on literature data about Bet v 1 isoforms and mutants, indicating that replacement of Bet v 1 S112 with C led to a reduction of IgE binding capacity, and on initial IgE binding tests of our Bet v 1 clone B2 containing P in positions 112 and 151 which revealed a very low IgE reactivity of the expressed protein. Consequently, S111C and S111P mutants were generated from the two Mal d 1 isoallergen clones GD26 and GS29. The protein sequence of GD26 was 99.4 % identical with Mal d 1a, whereas GS29 was 99.4 % identical with Mal d 1b. Both wild type clones differed in 14 amino acid residues (90.6 % sequence identity). The resulting Mal d 1 mutants were named GDC, GDP, GSC and GSP, respectively. In the case of Bet v 1, clone B2 was used as template and mutated stepwise to obtain allergens corresponding to the highly allergenic Bet v 1a and mutants containing C or P in position 112. For this purpose, two point mutations had to be introduced into the B2 sequence resulting in the following clones: B2S (B2 P112S, P151L) and B2C (B2 P112C, P151L). The amino acid sequence of B2S is identical with Bet v 1a. B2P (B2 P151L), another single point mutant of B2 differed from Bet v 1a only in S112 replaced with P. All mutated nucleotide sequences were cloned into pGEM-T and confirmed by sequencing.

Expression and purification of recombinant allergens and mutants

The procaryotic expression vector pET-15b encodes for an N-terminal fusion peptide of 21 amino acids, containing a 6-residue His-Tag followed by a thrombin cleavage site. Hence, the Mal d 1 fusion proteins consisted of 179 amino acids with a molecular mass of approximately 21 kDa. The Bet v 1 fusion proteins contained 180 amino acids with a mass similar to Mal d 1. As shown for all mutated allergens in Fig. 4, a strong protein expression

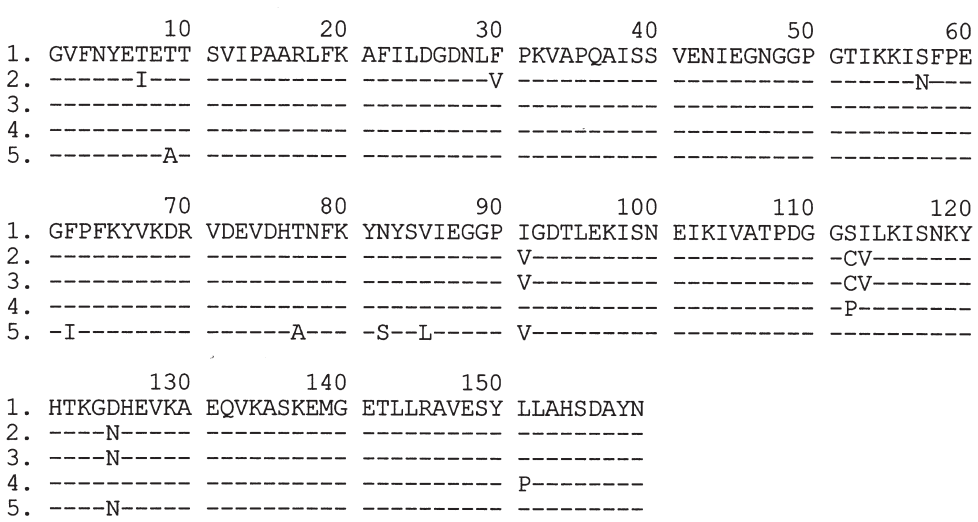

Fig. 3 Comparison of Bet v 1 isoallergen sequences. Residues identical with Bet v 1a are marked by "-". (1) Bet v 1a, (2) Bet v 1d, (3) B1, (4) B2, (5) B3.

Table 1 Degree of amino acid sequence identity between Bet v 1 and Mal d 1 isoforms

	Bet v 1a	Bet v 1d	B1	B2	B3	Mal d 1a	Mal d 1b	Mal d 1c
Bet v 1a	100	95.6	97.5	98.8	95.6	56.2	56.9	56.2
Bet v 1d		100	98.1	95.0	93.8	56.2	55.6	56.2
B1			100	96.9	95.6	56.2	55.6	56.2
B2				100	94.4	55.6	56.2	55.6
B3					100	58.8	57.5	58.8

was obtained with the plasmids containing an insert, whereas no expression occurred without induction or with cells transformed with the vector without insert. The Mal d 1 fusion proteins appeared at about 21 kDa, whereas the Bet v 1 fusion proteins had an apparent mass of approximately 20 kDa. The time-depended expression was monitored by SDS-PAGE, revealing that a plateau of specific expression was reached 4-5 h after induction (not shown). Therefore, to obtain a sufficient amount of recombinant allergen, 400 ml cultures were induced for 6 h. Since the recombinant allergens were almost completely found in the insoluble inclusion body fraction, the cell pellets were lysed under denaturing conditions and purified by Ni-chelate affinity chromatography. Between 2.5 and 7.5 mg of recombinant allergens and mutants were isolated from 100 ml of bacterial culture. After elution, the samples contained 6 M urea. During stepwise removal of urea by dialysis, all fusion proteins precipitated at urea concentrations between 2-4 M, thus making enzymatic cleavage of the His-Tag in solution impossible. To circumvent this problem, the fusion proteins were loaded onto Ni-sepharose column, the urea was removed stepwise and the column was flushed with thrombin in binding buffer. After overnight incubation, the non-fusion proteins were eluted stepwise with binding buffer and with the buffer containing 6 M urea. Fractions were collected and analysed by SDS-PAGE. As shown in Fig. 5, pure non-fusion allergens either eluted in pure binding buffer or in the fraction containing urea. As shown in lane 5 of Fig. 5, for some allergens, e.g. GS29 and GD26, a major proportion of the fusion protein remained undigested, and proteolytic fragments of about 14 kDa resulted from the overnight incubation with protease. The overall cleavage rates ranged between 30 % and 60 %. To obtain a higher yield, undigested non-fusion proteins were subjected to a second round of proteolytic treatment. The Mal d 1 non-fusion proteins had an apparent mass of 18 kDa whereas non-fusion Bet v 1 presented a molecular weight of 17 kDa, both corresponding well to the size of the natural allergens (3). In summary the wild type Mal d 1 clones GD26 and GS29, corresponding to Mal d 1a and Mal d 1b, were selected for expression, as were our Bet v 1 clones B1 and B2, the first being highly identical with Bet v 1 d,

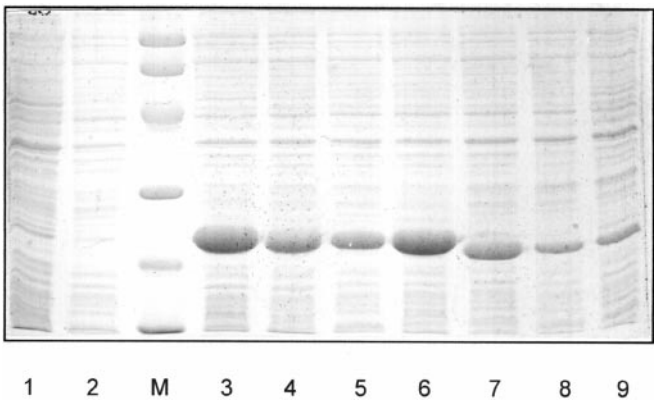

Fig. 4 Expression of recombinant mutated allergens in *E.coli* controlled by SDS-PAGE. Five µl of lysed bacteria were applied. Lanes: (1) *E.coli* BL21 (DE3) transformed with vector pET-15b without insert, (2) bacteria transformed with GDP without induction, lanes (3)-(9) induction with IPTG for 6 h, (3) GDP, (4) GDC, (5) GSP, (6) GSC, (7) B2S, (8) B2C, (9) B2P. The gel was stained with Coomassie brilliant blue.

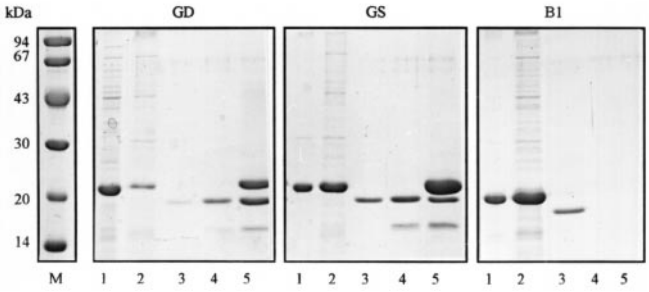

Fig. 5 Elution of non-fusion proteins after on-column thrombin digestion of recombinant apple and birch pollen allergens. (1) Cell pellet dissolved in binding buffer containing 6 M urea (2.5 µl), (2) cell lysate after column passage (10 µl), (3) binding buffer eluate after overnight treatment with thrombin (4), eluate obtained with binding buffer containing 6 M urea (15 µl), (5) eluate obtained with binding buffer containing 6 M urea and 1 M imidazole. Allergens as indicated. The gel was stained with Coomassie brilliant blue.

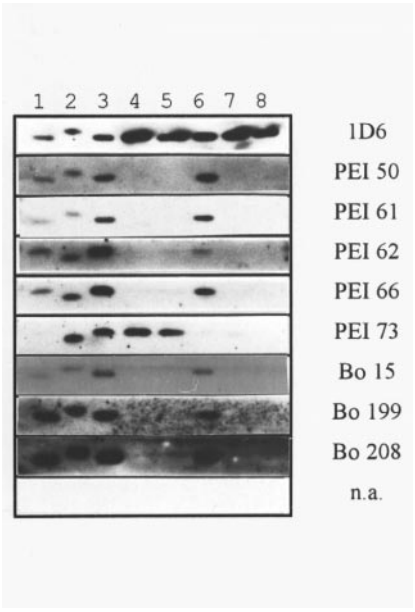

Fig. 6a Immunoreactivity of Mal d 1 isoallergens and mutants investigated by immunoblotting of sera from 8 patients allergic to birch pollen and apples, a non allergic control serum (n.a.) and mab 1D6 raised against natural Mal d 1. Lanes: (1) natural Mal d 1, (2) Mal d 1 c, (3) GD26, (4) GDP, (5) GDC, (6) GS29, (7) GSC, (8) GSP. Amount of allergen 0.1 µg. Serum dilution 1:20.

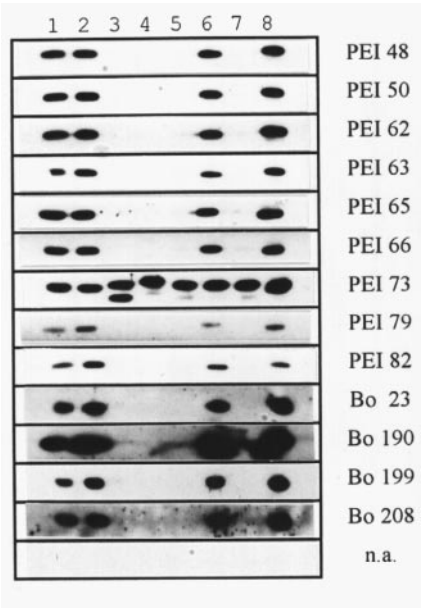

Fig. 6b Immunoreactivity of Bet v 1 isoallergens and mutants investigated by immunoblotting of sera from 13 patients allergic to birch pollen and apples and a non allergic control serum (n.a.). Lanes: (1) natural Bet v 1, (2) Bet v 1a, (3) B1, (4) B2, (5) B2P, (6) B2S, (7) B2C, (8) Bet v 1a M139L. Amount of allergen 0.1 µg. Serum dilution 1:20.

and the latter encoding for an isoform that showed 2 proline exchanges when compared to the high IgE-binding Bet v 1a. Moreover, 4 Mal d 1 mutants, GDC, GDP, GSC, GSP, and 3 Bet v 1 mutants B2S (=Bet v 1a), B2P and B2C were expressed, purified, and subjected to allergenicity testing as non-fusion proteins.

IgE binding capacity of recombinant allergens and mutants investigated by immunoblotting

The first series of experiments was based on comparative immunoblotting. Natural Mal d 1 and Bet v 1 were purified by preparative SDS-PAGE and included in these experiments. Mal d 1c and Bet v 1a are commercially available and were also included. The mutant Bet v 1a M139L had been used for structural investigations by nuclear magnetic resonances revealing that this point mutation did not affect the tertiary fold of the allergen (30). All allergens and mutants were subjected to SDS-PAGE and transferred to NC membranes (0.1 µg allergen were applied in a 3 mm analytical slot). The 16-18 kDa regions of the NC were excised and probed with sera from patients with concomitant allergies to birch pollen and apples. Mal d 1 allergens were further tested with mab 1D6 raised against purified natural Mal d 1. In the case of Mal d 1, 8 sera from allergic patients and serum from a non-allergic volunteer were used (Fig. 6a). Sera from all allergic patients showed IgE binding to GD26 (lane 3) and Mal d 1 c (lane 2). Seven of the allergic patients had IgE recognising GS29 (lane 6). Natural Mal d 1 (lane 1) also bound IgE from seven of the patients, but was only weakly detected by IgE from patients PEI 61 and Bo 15 (lane 1). The mutants GSC and GSP were not IgE-reactive in the immunoblot assay (lanes 7 and 8). This was also true with GDC and GSP (lanes 4 and 5), with the exception of patient PEI 73 whose IgE antibodies bound strongly to all GD26-derived allergens and to Mal d 1c, but did not recognise any of the other samples. No reactivity occurred with the non-allergic control serum, whereas mab 1D6 bound strongly to all allergens and mutants.

In the case of Bet v 1, sera from 13 allergic patients and a non-allergic control were used for immunodetection (Fig. 6b). The IgE reactivity to natural Bet v 1 (lane 1) was equal to that of Bet v 1a (lane 2), B2S (lane 6) and Bet v 1a M139L (lane 8). The recombinant isoforms B1 and B2 (lanes 3 and 4) as well as the mutants B2P and B2C (lanes 5 and 7) were not IgE-reactive except for patient PEI73 whose IgE antibodies bound strongly to all Bet v 1 allergens and mutants. Isoform B1 appeared as a double band, possibly caused by a proteolytic cleavage product. No IgE binding occurred with the non allergic control serum. During the EAST measurements described below, mutant B2C presented a significantly higher IgE binding capacity than B2P. To visualise these differences by immunoblotting, the protein concentration of the mutants B2S, B2P and B2C was increased fourfold to 0.4 µg per slot, and the samples were proc-

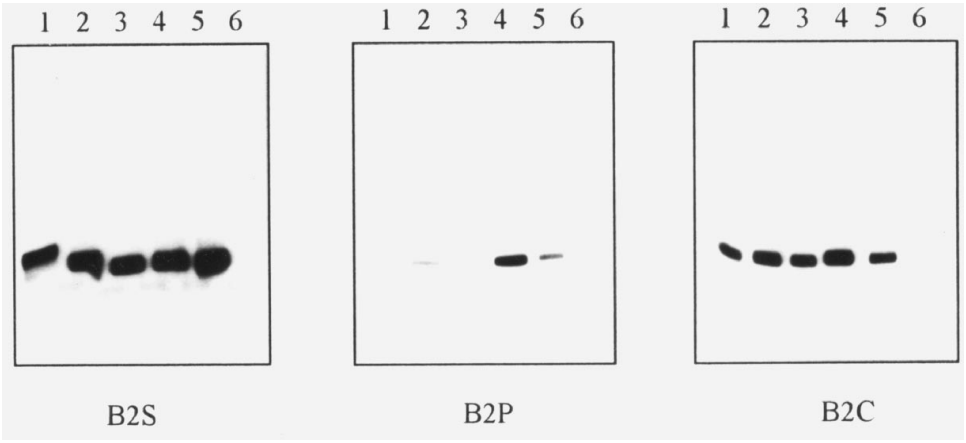

Fig. 7 Immunoreactivity of Bet v 1 mutants investigated at fourfold increased protein concentration by immunoblotting of sera from 5 patients allergic to birch pollen and apples and a non-allergic control serum. Sera: (1) Bo 23, (2) Bo 190, (3) Bo 199, (4) Bo 208, (5) PEI 66, (6) non-allergic control, Allergens B2S, B2C and B2P as indicated. Serum dilution 1:20.

essed by SDS-PAGE and semi-dry blotting to NC. Five sera from the allergic patient panel as well as serum from a non-allergic control were used for immunodetection (Fig. 7). Under these experimental conditions, B2S which is identical with Bet v 1a bound strongly to IgE from all allergic patients, and B2C was also IgE reactive with all sera, but the intensity of staining was clearly decreased. Mutant B2P showed negligible IgE binding with 4/5 sera and thus presented the weakest IgE binding capacity.

IgE binding capacity of recombinant allergens and mutants investigated by enzyme allergosorbent test

To verify the results of immunoblotting by an assay procedure that is carried out under more native conditions, extracts and recombinant allergens were coupled to activated filter paper disks and incubated with sera from 13 patients with birch pollen related apple allergy. The resulting EAST classes, indicating semi-quantitatively the amount of allergen-specific IgE are summarised in Table 2 for apple allergens and in Table 3 for birch pollen allergens.

Apart from slight differences, apple extract, natural Mal d 1, Mald 1c, and GD26 presented similar IgE binding capacity, whereas the IgE reactivity of GS29 was slightly weaker. The GDC and GSC mutants were clearly IgE-reactive with a slight reduction measured with some sera. By contrast, mutants GDP and GSP presented a drastic decrease of IgE reactivity with sera from almost all apple-allergic patients (Table 2). With birch pollen allergens, the IgE reactivity was very high to birch pollen extract, natural Bet v 1, and Bet v 1a (Table 3). The isoform B1 showed a clearly decreased IgE binding capacity with EAST classes of 0-2, whereas the highly reactive allergens presented classes of 3-4. The allergens derived from clone B2 showed results that closely corresponded to immunoblotting: B2 was not IgE-reactive with the exception of serum PEI 73 which produced a class 2 result and PEI 45 with a class 1 result. This weak reactivity was also preserved for mutant B2P for which slightly increased EAST-classes were determined. By contrast, introduction of cysteine or serine in position 112 of Bet v 1 reestablished the full allergenicity.

Cross-linking of cell-bound IgE by RBL cell mediator release assay

Cross-linking of mast cell bound IgE and subsequent mediator release are essential events of the type I allergic reaction. This property was evaluated by the RBL cell me-

Table 2 IgE binding capacity of Mal d 1 allergens and mutants determined by EAST with sera of 13 apple-allergic patients with birch pollinosis

EAST-class	apple extract	nMal d 1	Mal d 1c	26 GD	GDC	GDP	29 GS	GSC	GSP
0	0	0	0	0	1	13	0	2	10
1	0	0	1	1	2	0	1	2	1
2	5	8	5	4	8	0	6	5	2
3	8	5	7	8	2	0	4	4	0
4	0	0	0	0	0	0	0	0	0

Numbers in the allergen columns indicate number of sera with the respective EAST class to the allergen/extract.

Table 3 IgE binding capacity of Bet v 1 allergens and mutants determined by EAST with sera of 13 apple-allergic patients with birch pollinosis

EAST-class	birch extract	nBet v 1	Bet v 1a	B1	B2	B2P	B2C	B2S
0	0	0	0	1	11	7	0	0
1	0	0	0	5	1	3	0	0
2	0	0	0	6	1	3	0	0
3	6	4	4	1	0	0	7	6
4	7	9	9	0	0	0	6	7

Numbers in the allergen columns indicate number of sera with the respective EAST class to the allergen/extract.

diator release assay which has been developed as a murine model of the type I reaction (26). RBL 2H3 cells were passively sensitised with a murine reaginic serum pool raised against birch pollen extract. Recombinant and natural apple and birch pollen allergens were used for dose-related stimulation of the sensitised cells. As depicted in Fig. 8a, natural Mal d 1 and isoform GD26 possessed the highest biological activity in this assay system, eliciting more than 60 % mediator release at a stimulator concentration of 1 µg/ml. GS29 and Mal d 1c had a moderate activity with about 20 % mediator release at 1 µg/ml. The four mutants GDC, GDP, GSC and GSP were weakly active with a maximal release of less than 20 % at a concentration of 10 µg/ml.

Stimulation with Bet v 1 resulted in similarly shaped release plots for nBet v 1, Bet v 1a, the B2S-mutant which had the Bet v 1a protein sequence, and B2C. However, different maximal releases were measured at 10 ng/ml which ranged from 35 % for nBet v 1 and 25 % for Bet v 1a to approximately 15 % for B2S and B2C (Fig. 8b). B1 had an about 10-fold decreased activity, with a maximal mediator release of 21 % at 1 µg/ml. B2P and B2 had a very weak allergenic potency, being about 100-1000-fold reduced in relation to nBet v 1, Bet v 1a, B2S and B2C.

Expression of Mal d 1 in apples from different varieties

Protein extracts were prepared from the same apples that had been used as source material for the cloning of Mal d 1. Equal amounts of protein from the seven varieties were subjected to analytical SDS-PAGE, and the gel was stained with coomassie brilliant blue. The calculation of the Mal d 1 content was based on the total soluble protein extracted from 100 g of apple determined by a dye binding assay in combination with taking the total electrophoretic protein pattern as 100 % level for estimating the relative Mal d 1 amount in the respective extract. Ex-

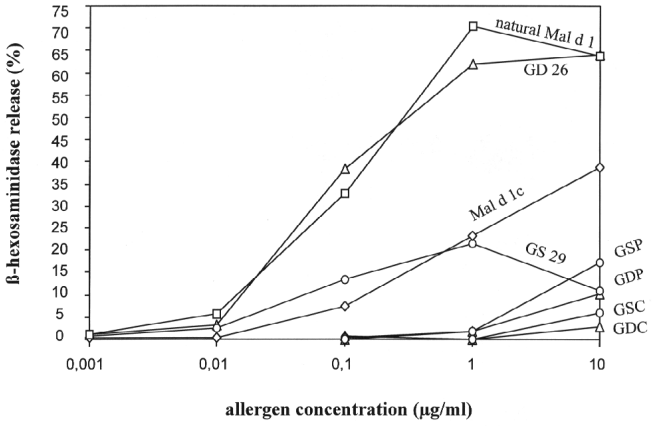

Fig. 8a Mediator release from RBL 2H3 cells passively sensitised with murine IgE pool against birch pollen extract. The amount of released β-hexosaminidase was assayed by a colorimetric reaction. Apple allergens were used as elicitors as indicated.

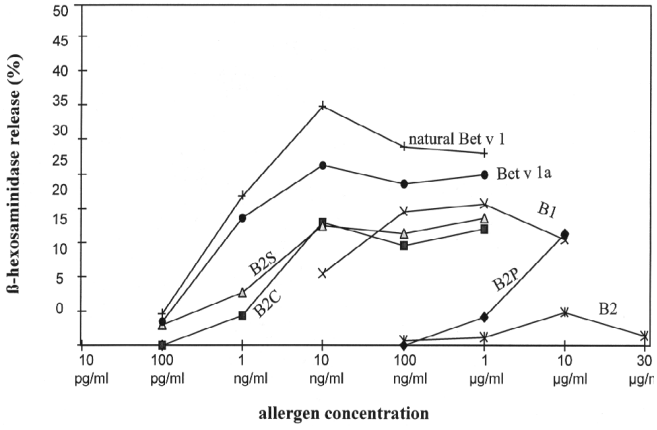

Fig. 8b Mediator release from RBL 2H3 cells passively sensitised with murine IgE pool against birch pollen extract. The amount of released β-hexosaminidase was assayed by a colorimetric reaction. Birch pollen allergens were used as elicitors as indicated.

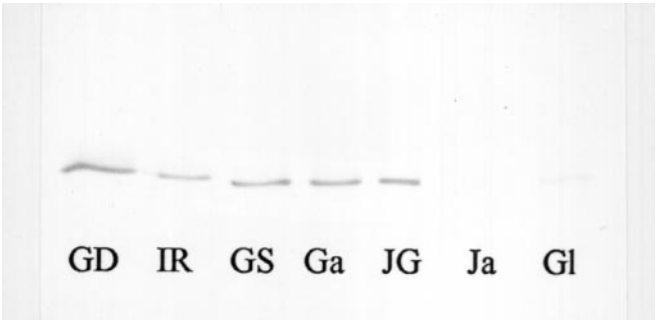

Fig. 9 Identification of Mal d 1 in extracts from apples belonging to different varieties by immunoblotting. The mab 1D6, raised against natural Mal d 1 was used as primary probe. GD: Golden Delicious, IR: Idared, GS: Granny Smith, Ga: Gala, JG: Jonagold, Ja: Jamba, Gl: Gloster.

pressed as mg/100 g fresh apple, the following Mal d 1 levels were determined: Golden Delicious: 4.5, Granny Smith: 1.6, Jonagold: 0.7, Idared: 0.8, Royal Gala: 1.8, Jamba: 0.5, Gloster: 0.4. The occurrence of Mal d 1 in these extracts was confirmed by immunoblotting with the mab 1D6 (Fig. 9). A Mal d 1 band was clearly detected in the five strains with the highest Mal d 1-content, whereas samples from the strains Jamba and Gloster which contained the lowest Mal d 1-amount according to the analysis of the total protein pattern lacked antibody binding ability.

Discussion

The aim of this study was to investigate the molecular basis of differences in the allergenic potency between apples from different varieties, and to get insights in the nature of the cross-reactive epitopes on the major allergens of birch pollen and apple.

The apple strains used as source material for this investigation were selected on the basis of patient's reports and previous results (9). For example, Golden Delicious and Granny Smith have been described as highly allergenic, whereas Jamba and Gloster were tolerated by two apple allergic patients and extracts from these strains weakly reacted with apple-specific IgE (9). Since the applied PCR strategy was based on regions shared by the cDNAs of the three known Mal d 1 sequences by selecting the consensus primers AM4 and MMU, it is likely that our amplification protocol was suitable to amplify the majority of Mal d 1 isoforms. By 5'RACE the sequence of the 5'-end primer AM5 was identified in the cDNA of Mal d 1 from all apple varieties, thus confirming AM5 as a strictly conserved partial sequence. When compared with the Mal d 1c sequence (6) AM5 presents one nucleotide exchange although the primer MMU used in the PCR of 5'RACE products exactly matched with the corresponding segment of Mal d 1c. This may be seen as further evidence for the view that the Mal d 1c sequence is of minor importance among the major allergen isoforms expressed by apple fruits. Sequencing of PCR products obtained with AM5 and oligo-dT as primers confirmed that the AM4 primer sequence was identical in all PCR products.

Our results clearly showed that mRNA of Mal d 1 was present in apples from all seven strains. On the contrary, analysis of the protein pattern revealed more than tenfold differences in the expression of the allergenic protein, ranging from 4.5 mg per 100 g of apple for Golden Delicious to 0.4 mg per 100 g for Gloster. The ranking of allergen expression found in this study correlates well with our previous results about the abundance of the major allergen in apple samples from different varieties (9, 19). Sequencing of the PCR products revealed the existence of two major groups of isoforms: Six clones were highly homologous to Mal d 1a (7) with identities between 97.5 % and 99.4 % at the amino acid level. Another six clones were almost identical with Mal d 1b (8), showing identities between 99.4 % and 100 %. The identity between these two groups was about 90 %. The third known isoform, Mal d 1c (6) showed specific sequence deviations to all sequences obtained in our study. We therefore conclude that this isoform is of minor importance.

The occurrence of mRNA of Mal d 1 isoallergens is not strain-specific, and more than one isoform can be present in a specific fruit. This can be concluded from the result that mRNA of both the Mal d 1a and the Mal d 1b group were found in the Golden Delicious, Gala and Gloster samples. Moreover, two clones obtained from Granny Smith PCR products contained the Mal d 1b sequence, originally found in Golden Delicious. In contrast, the Mal d 1a sequence has originally been cloned from Granny Smith.

As determined by immunoblotting, EAST and RBL cell mediator release the allergenic potency of Mal d 1a, represented by our recombinant allergen GD26, appears to be higher than that of Mal d 1b, corresponding to our allergen GS29. Even more drastic differences between isoforms have been reported for Bet v 1 (10). However, it is likely that both Mal d 1 isoforms are expressed in parallel and thus drastic differences in the allergenic activity of apples cannot be explained by the existence of these isoforms. However, once both isoforms are expressed in parallel in one fruit the relative concentrations of Mal d 1a and Mal d 1b may determine minor differences in the allergenicity between strains. In conclusion, the only remaining reason for major differences in the allergenic potency of apples are deviating expression levels. This view correlates well with the protein patterns found in the apple samples that were used for RNA preparation, and with our immunoblotting results (Fig. 9). Northern blotting

with a Mal d 1-specific probe would be a suitable method to confirm this hypothesis and possibly, to analyse the expression levels of the two major isoforms. However, the amount of extractable total RNA was to low to apply this method.

To compare Mal d 1 with its highly allergenic homologue from pollen, the cloning of Bet v 1 was included in this study. Although the gene specific primers were designed from the cDNA of the highly allergenic Bet v 1a (22), none of our three Bet v 1 clones presented a sequence completely identical with Bet v 1a. Isoform B1 was 98.1 % identical with the low IgE binding isoform Bet v 1d (10-12) and contained a characteristic CV-motif in position 112/113 which has been used to create low allergenic mutants of Bet v 1 (29). Interestingly, the cloned isoform B2 also contained S112 of Bet v 1a replaced with another amino acid, namely proline. Since our clone B3 contained a mixed sequence of Bet v 1 isoallergens, we decided to focus on the expression of B1 and B2 and possibly to identify a hot spot for IgE reactivity. Indeed, B1 had a reduced and B2 an almost depleted IgE reactivity when compared to Bet v 1a (Fig. 6b, Table 3). To further investigate the importance of S112 for IgE binding and for the cross-reactive epitope on Mal d 1, site directed mutagenesis was applied. In the case of Bet v 1, clone B2 was used as template and stepwise mutated to the Bet v 1a sequence. The drastic differences between the IgE binding to mutated allergens B2P and B2S which differed in one amino acid only demonstrated that proline in position 112 is a key residue for 'hypoallergenicity'. By contrast, as shown by the results with mutant B2C, C at this position had a weaker influence of the overall allergenicity. These data were confirmed by introducing C and P at the corresponding position of S111 in the two Mal d 1 isoforms GD26 and GS29 (Fig. 6a, Table 2). Again, the IgE reactivity decreased from the wild type S111 via C111 to the P111 mutants. In conclusion, our mutation studies show that positions 112 in Bet v 1 and 111 in Mal d 1 are 'hot spots' for IgE reactivity, and that the cross reactive epitope can be removed by a single point mutation. Structural analyses have shown that this key residue is the first in a β-sheet, and that it is exposed on the surface of both Bet v 1 and the Mal d 1-related cherry allergen Pru a 1 (31). Hence, it is possible that serine at this position is a part of an epitope. On the other hand, proline is known to cause major structural changes in the protein fold, and the drastically decreased IgE reactivity of the proline mutants may also reflect major changes in the tertiary fold of the allergens. Further investigations are required to answer this question.

Our finding that allergenic differences are mainly related to expression levels of Mal d 1 may initiate studies at the genomic level. Knowledge about promoter regions may be helpful to identify markers for low level expression which might be useful for breeding approaches targeting reduced allergenicity. The restricted number of highly homologous isoforms of Mal d 1 identified in this study may offer the possibility of applying antisense RNA approaches for Mal d 1 suppression in apple plants. Finally non-IgE binding isoforms of pollen allergens that contain the sequential epitopes recognised by allergen-specific T-cells have been suggested as improved therapeutic allergy vaccines with reduced side effects (29). The B2 allergen cloned and characterised in this investigation may be useful for such approaches.

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